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(54) Title: INDUCIBLE PLANT DEFENSE GENE REGULATORY REGIONS FROM POTATO AND RICE, USES THEREOF, AND ASSAYS

(57) Abstract

The present invention provides isolated regulatory sequences or promoters for plant genes that encode phenylalanine ammonia-lyase (PAL) in potato or rice. The promoters are inducible by exogenous elicitor(s) and/or wounding, and are capable of regulating transcription of associated DNA sequences in chimeric plant cells, tissues and plants that contain the chimeric constructs. The invention provides chimeric potato and rice PAL constructions, transgenic potato and rice plant cells, plant tissues and transgenic plants that contain them. The chimeric constructions and transgenic plant materials are useful in agrichemical assays. The invention also provides a method for identifying inducible plant defense genes and promoters. In addition, the invention provides a method for identifying agrichemicals that can induce plant defense genes, especially agrichemicals which induce these plant gene regulatory elements immediately or very soon after exposure. Finally, the invention provides a method for identifying organisms which can induce plant defense gene regulatory elements.

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INDUCIBLE PLANT DEFENSE GENE REGULATORY REGIONS FROM POTATO AND RICE, USES THEREOF, AND ASSAYS

FIELD OF THE INVENTION

This invention relates generally to plant biotechnology and specifically to novel inducible plant defense gene regulatory sequences or promoters from potato and rice, methods for identifying and isolating these and related sequences, plus uses thereof, especially in the construction of transgenic plants and plant compositions. The invention also relates to methods for identifying agrichemicals and organism(s) which can induce plant defense genes.

BACKGROUND OF THE INVENTION

Plant disease resistance mechanisms include physical and chemical barriers within the plant, as well as responses which are induced or activated by wounding or attack by pathogen(s). One important inducible defense response is the production of phytoalexins, which have been defined as low-molecular weight, antimicrobial compounds synthesized and accumulated by the plant at the site of attempted infection. Phytoalexins are predominantly phenylpropanoids, isoprenoids, and acetylenes.

The first committed step in the biosynthesis of phenylpropenoids in higher plants is the formation of ammonia and *trans*-cinnamate from L-phenylalanine. This reaction is catalyzed by the enzyme L-phenylalanine ammonia-lyase (PAL). The organization of PAL genes has been most extensively studied in bean (*Phaseolus vulgaris*). *See* Cramer, *et al.*, 1989, *Plant Mol. Biol.* 12:367-383. In the bean genome, there are three divergent classes of PAL genes: gPAL-1, gPAL-2 and gPAL-3.

Transcription of PAL genes is rapidly activated by a wide range of stimuli including wounding, glycan elicitor preparations from fungal cell walls, and the reduced form of glutathione, a cellular metabolite. Not all PAL genes respond in the same manner to these stimuli. Therefore there is a need to identify novel PAL promoters which respond to fungal or bacterial elicitors, or to wounding. These novel PAL promoters can be used to create transgenic

plant materials and transgenic plants, and as part of novel agrichemical screening assays.

It is an object of the present invention to identify and provide novel PAL promoters which respond for example, to fungal or bacterial elicitors, or wounding. It is a further object of the invention to use these novel promoters to create transgenic plant materials and transgenic plants. It is another object to provide novel methods which will allow the new PAL promoters to be identified and isolated. It is yet another object of the invention to provide novel whole plant agrichemical screening assays and techniques useful for identifying new agrichemicals which induce the plant's normal PAL-related defense responses, and will also induce expression in transgenic plants of structural genes associated with responsive PAL promoters. It is also an object to provide an improved method for identifying agrichemicals which can induce plant defense gene promoters or regulatory regions, especially agrichemicals which induce these plant gene regulatory regions immediately or very soon after exposure. Finally, it is an object of the invention to provide a method for identifying organisms which can induce plant defense gene regulatory regions.

SUMMARY OF THE INVENTION

The present invention provides isolated promoters (regulatory sequences) for plant genes that encode phenylalanine ammonia-lyase (PAL) in potato or rice. The promoters are inducible by exogenous elicitor(s) or wounding, and are capable of regulating transcription of associated DNA sequences in transgenic plant cells, tissues and plants that contain the promoters as part of chimeric constructs. The invention includes methods for obtaining these novel PAL promoters. The invention provides the PAL promoter(s), probe sequences, chimeric potato and rice PAL promoter constructs (in which the PAL promoters are operatively linked to structural genes, *e.g.*, reporter genes), as well as transgenic potato and rice plant cells, plant tissues and transgenic plants that contain these chimeric constructs. The chimeric constructions and transgenic plant materials which contain them are useful in agrichemical assays. The invention also provides a method for

inducing transcription of a chimeric gene in transgenic plants. The invention further provides a method for identifying agrichemicals that can induce plant defense gene elements, especially agrichemicals which induce plant gene regulatory elements immediately or very soon after exposure. Finally, the invention provides a method for identifying organisms which can induce plant defense gene regulatory elements.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A and 1B are restriction enzyme maps of genomic clones of potato *S. tuberosum* PAL genes.

Figure 1A shows limited restriction enzyme maps of six potato clones isolated from the *S. tuberosum* cv. Desirée genomic library. Clone 1 = λpPAL-1; Clone 3 = λpPAL-3; Clone 4 = λpPAL-4; Clone 6 = λpPAL-6; Clone 7 = λpPAL-7; Clone 8 = λpPAL-8. The boxes indicate the regions of each clone that have been subcloned and that contain sequences which hybridize to *S. tuberosum* cv. Desirée cDNA clones pPAL-3 and pPAL-21. The orientation of the putative PAL gene is given within the boxes. Symbols: B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII; K: *Kpn*I; S: *Sst*I. The parentheses around some symbols indicate the restriction enzyme cleavage sites are present, but the exact location is not known.

Figure 1B also shows restriction enzyme maps of seven potato clones isolated from the *S. tuberosum* cv. Desirée genomic library. Clone 1 = λpPAL-1; Clone 2 = λpPAL-2; Clone 3 = λpPAL-3; Clone 4 = λpPAL-4; Clone 6 = λpPAL-6; Clone 7 = λpPAL-7; Clone 8 = λpPAL-8. The orientation of the putative PAL gene is given below each map. Symbols: B: *Bam*HI; Bgl: *Bgl*I; E: *Eco*RI; H: *Hin*dIII; P: *Pst*I; S: *Sst*I. The parentheses around some symbols indicate that the restriction enzyme cleavage sites are present, but the exact location is not known. (In the present specification, and with reference to the figures, the B→S sequence for λpPAL-1 is **SEQ ID NO. 1**; the (E)→P sequence for λpPAL-2 is **SEQ ID NO. 2**; the (E)→P sequence for λpPAL-3 is **SEQ ID NO. 3**; the E→P sequence for λpPAL-4 is **SEQ ID NO. 4**; the H→S sequence for λpPAL-6 in Figure 1B is referred to as λpPAL-6(a) and is shown as **SEQ ID NO. 5**; the S→H sequence (shown as a sense sequence,

having been converted from the antisense sequence) for λpPAL-6 is referred to as λpPAL-6(b) and is shown as SEQ ID NO. 6; and the (E)→P fragment for λpPAL-8 is SEQ ID NO. 7.)

Figure 2 shows restriction enzyme maps of genomic clones of the rice *Oryza sativa* PAL genes. More specifically, Figure 2 shows limited restriction enzyme maps of three rice PAL clones isolated from the *Oryza sativa* genomic library. Clone 2 = λrPAL-2; Clone 4 = λrPAL-4; Clone 10 = λrPAL-10. The scale is shown in the bottom left corner. The boxes indicate regions that have been subcloned into a pUC vector. The arrows above the maps show the orientation of the PAL gene within the genomic clone. Symbols: E: *Eco*RI; H: *Hin*dIII; S: *Sst*I. (In the present specification, λrPAL-2 in Figure 2 is SEQ ID NO. 8 and λrPAL-4 is SEQ ID NO. 9).

Figure 3 is a schematic drawing showing the self-sustained, sequence replication (3SR™, SIBIA, La Jolla, CA) system.

DEFINITIONS

In the present specification and claims, reference will be made to phrases and terms of art which are expressly defined for use herein as follows:

As used herein, PAL refers to phenylalanine ammonia-lyase. PAL catalyzes the conversion of the amino acid L-phenylalanine to *trans*-cinnamic acid and NH_4^+. This is the first reaction in the synthesis of a wide range of plant natural products based on the phenylpropane skeleton, including lignins, flavonoids, isoflavonoid, coumarins and hydroxycinnamic acid esters.

As used herein, exogenously controlled plant gene regulatory regions or elements refer to nucleic acid sequences that affect transcription of "operatively linked, functionally linked or associated" structural genes in response to exogenous stimuli. Examples of exogenously controlled plant gene regulatory sequences include inducible plant defense gene regulatory regions or promoters (*e.g.*, PAL promoters), elicitor-regulated activator domains, upstream silencer domains, *etc.*

As used herein, promoter refers to a non-coding region of DNA involved in binding of RNA polymerase and other factors that initiate or modulate transcription whereby an RNA transcript is produced. Promoters

can be naturally occurring or synthetically produced. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. A constitutive promoter is always turned on. A regulatable promoter requires specific signals in order for it to be turned on or off. A developmentally regulated promoter is one that is turned on or off as a function of development. The promoters of the present invention are directly or indirectly responsive to exogenous elicitor(s) and/or wounding. Stated another way, exogenous elicitor(s) and/or wounding can induce the promoters of the present invention to initiate transcription of associated structural gene(s).

In the present specification and claims, the terms promoter and gene regulatory region are used interchangeably.

As used herein, elicitors are compounds or organisms which control (*e.g.,* initiate, terminate, increase or reduce), either directly or indirectly, the action of exogenously-controlled plant gene regulatory elements such as the inducible stress-regulated promoters for the plant genes that encode the phenylpropanoid biosynthetic enzymes phenylalanine ammonia-lyase (PAL). Elicitors include, but are not limited to, the reduced form of glutathione; the reduced form of homoglutathione and the reduced form of other peptide analogs of glutathione; glycan elicitors such as hexa(β-D-glucopyranosyl)-D-glucitols; lipid elicitors such as arachidonic acid and eicosapentaenoic acid, glycoprotein elicitors, fungal elicitors, abiotic elicitors such as mercuric chloride $HGCl_2$, fungi (*e.g.,* elicitors released by whole fungi), bacteria (*e.g.,* elicitors released by whole bacteria), *etc.*

As used herein, the terms operatively linked, functionally linked or associated, or grammatical variations thereof, are equivalent terms that are used interchangeably. In particular these terms refer to the linkage of a promoter or a non-coding gene regulatory sequence to an RNA-encoding DNA sequence, and especially to the ability of the regulatory sequence or promoter to induce production of RNA transcripts corresponding to the DNA-encoding sequence when the promoter or regulatory sequence is recognized by a suitable polymerase. All three terms mean that linked DNA sequences (*e.g.,* promoter(s), structural gene (*e.g.,* reporter gene(s)), terminator sequence(s),

etc.) are operational or functional, *i.e.,* work for their intended purposes. Stated another way, operatively or functionally linked, or associated, means that after the respective DNA segments are joined, upon appropriate activation of the promoter, the structural gene will be expressed.

As used herein, suitable plant material means and expressly includes, plant protoplasts, plant cells, plant callus, plant tissues, developing plantlets, immature whole plants and mature whole plants.

As used herein, transgenic plants or plant compositions refer to plants or plant compositions in which heterologous or foreign DNA is expressed or in which the expression of a gene naturally present in the plant has been altered. Such DNA will be in operative linkage with plant biochemical regulatory signals and sequences. Expression may be constitutive or may be regulatable. The DNA may be integrated into a chromosome or integrated into an episomal element, such as the chloroplast, or may remain as an episomal element. In creating transgenic plants or plant compositions, any method for introduction of such DNA known to those of skill in the art may be employed.

Use of the phrase isolated in the present specification and claims, as a modifier of DNA or RNA, means that the DNA or RNA so designated have been separated from their *in vivo* cellular environments through the efforts of human beings; as a result of this separation, the isolated DNAs or RNAs are useful in ways that the non-separated, impure DNAs or RNAs are not.

DESCRIPTION OF SPECIFIC EMBODIMENTS

In one aspect, the present invention comprises isolated or isolatable promoters (*i.e.,* regulatory sequences) for plant defense genes that encode phenylalanine ammonia-lyase (PAL) in potato or rice wherein the promoters are capable of regulating transcription of an associated DNA sequence in suitable hosts and wherein the promoters can be induced by, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) and/or wounding. These promoters are exemplified herein by the potato PAL promoters associated with the PAL structural gene sequences comprised within

clones λpPAL-1, λpPAL-2, λpPAL-3, λpPAL-4, λpPAL-6, λpPAL-7 (λpPAL-6 and λpPAL-7 are identical as described in Example IIC and are referred to herein as λpPAL6/7), and λpPAL-8, plus rice PAL promoters comprised within clones λrPAL-2 and λrPAL-4.

In another aspect, the potato and rice PAL promoters of the invention are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait. The linkage can be at the level of transcription (*i.e.*, a "transcriptional fusion") so that the protein which gives rise to the phenotypic trait is expressed as a non-fused peptide. Alternatively, and especially in the case of some marker genes, the linkage can also be at the level of translation (*i.e.*, a "translational fusion") such that the marker protein is expressed as fusion peptide with a portion of the amino terminal end of the native PAL protein. According to the teaching of the invention, the phenotypic trait can consist of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, or production of an enzyme or reporter compound. When the associated structural gene encodes a reporter enzyme or compound, preferably the compound will be chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), or luciferase (LUX).

According to the invention, the potato and rice PAL promoters - associated DNA sequence constructs are used to transform suitable hosts and to create transgenic plant compositions, *e.g.*, plant protoplasts, plant cells, plant callus, plant tissue, developing plantlets, immature whole plants, mature whole plants, or seeds. The invention encompasses these transformed hosts and transgenic compositions, especially transgenic potato and rice plants and seeds that contain potato and rice PAL promoters and associated DNA sequences.

The present invention provides novel isolated DNA sequences identified herein as **SEQ ID NOS. 1-9**. **SEQ ID NOS. 1-7** are DNA coding sequences from potato PAL clones λpPAL-1, λpPAL-2, λpPAL-3, λpPAL-4,

λpPAL-6 (two sequences) and λpPAL-8, respectively. **SEQ ID NOS. 8 and 9** are coding and non-coding DNA sequences from rice PAL clones λrPAL-2 and λrPAL-4, respectively.

The DNA sequences of the invention can be used as probes to identify identical or homologous PAL sequences. Preferably the probes will be at least 10 nucleotides in length, and most preferably will be from about 100 to about 500 nucleotides long. For example, the coding sequences of potato PAL clones λpPAL-1, λpPAL-2, λpPAL-3, λpPAL4, λpPAL-6/7 or λpPAL-8, or λrPAL-2 or λrPAL-4 can be used to probe cDNA or genomic libraries for identical or highly homologous sequences. These identical or highly homologous sequences will be associated with inducible promoters that are well within the scope of the present invention.

Even though related PAL coding sequences may have sufficient homology to hybridize with one another under moderately stringent hybridization conditions, the promoters may not be functionally equivalent. For that reason the invention includes a method for identifying sequences that are not only homologous to the sequences of the invention, but are also regulated by inducible PAL promoters. According to this method, a plant is subjected to an elicitor, and then RNA from the plant is isolated. PolyA$^+$ RNA is selected, *e.g.*, by using an oligo(dT) column. A cDNA library is prepared from this RNA and cloned into a suitable vector. The library is probed with a probe comprised of nucleotide sequences from the coding region(s) of elicitor inducible genes, *e.g.*, the coding sequences of **SEQ ID NOS. 2-7** (λpPAL-2, λpPAL-3, λpPAL-4, λpPAL-6, λpPAL-8), or **SEQ ID NOS. 8 and 9** (λrPAL-2 and λrPAL-4). cDNA which hybridizes with the probe (*i.e.*, positive clone(s)) is subcloned and sequenced. A genomic library from the plant of interest is probed with the cDNA, and the segment of genomic DNA that hybridizes with the probe is identified. An antisense RNA transcript from this genomic DNA is made and labeled (*e.g.*, radioactively) and then used as a probe to hybridize to mRNA from elicitor-treated and non-elicitor treated plants. The hybridizing mix is then subjected to RNase degradation of all single-stranded RNA. If the transcript of interest is present

in total RNA, then a double-stranded RNA-RNA hybrid that is protected by RNA from elicitor-treated plants but that is not protected by RNA from non-elicitor containing no mismatches will be formed and will be unaffected by the RNase treatment. This product can be identified by size following gel electrophoresis. The promoter from the genomic clone that yields the antisense RNA transcript that is protected by RNA from elicitor-treated plants but that is not protected by RNA from non-elicitor treated plants is inducible with elicitor. The promoter is isolated and used to make chimeric constructs for use in agrichemical assays, and to produce transformed cells and transgenic plants and transgenic plant compositions.

The invention also includes a method for identifying exogenous elicitors which are capable of inducing, either directly or indirectly, a potato or rice PAL promoter. According to the method, a suitable host (*e.g.*, a plant composition) is transformed with a potato or rice PAL promoter operatively linked to a structural gene whose expression can be detected (*e.g.*, a marker gene). Putative exogenous elicitor is then applied to the transformed host. Exogenous elicitor that can induce expression of the marker gene is concluded to be an elicitor that can induce the potato or rice PAL promoter. In a related method, whole plants are used in the assay. According to this method, whole transgenic plants are created which contain at least one chimeric DNA sequence comprised of a potato or rice PAL promoter operatively linked to a reporter structural gene. Putative exogenous elicitor(s) is then applied to the plant. The elicitor(s) that induces expression of the reporter gene is concluded to be elicitor(s) which can induce expression of the potato or rice PAL promoters.

The invention also provides a method for identifying elicitor-inducible promoters. According to the method, a plant is subjected to an elicitor, then RNA is isolated from the plant. A cDNA library is prepared from the isolated RNA, which is then probed with a probe comprised of nucleotide sequences from the transcribed coding and/or non-coding region(s) of gene(s) of interest. cDNA which hybridized with the probe is then used to probe a genomic library from the plant of interest. The segment of genomic

DNA that hybridzed with the probe is identified, and a labeled antisense RNA transcript is made from it. This labeled antisense RNA transcript is used as a probe to hybridize to mRNA from elicitor-treated and non-elicitor treated plants. The hybridized mix is subjected to RNase, and those antisense RNA transcripts that were protected from degradation by elicitor-treated RNA but were not protected from degradation by RNA from non-elicitor treated plants are identified. The promoter from the genomic clone which yielded the antisense RNA transcript that was protected by only RNA from elicitor-treated plants is inducible with elicitor.

The invention also discloses a related method for identifying elicitor-inducible promoters. According to this method, a plant genomic library is probed with a probe comprised of nucleotide sequences from the transcribed coding and/or non-coding region(s) of a gene of interest. The segment of genomic DNA that hybridized with the probe is identified. A labeled antisense RNA transcript is made from the genomic DNA segment that hybridized with the probe. This labeled antisense RNA is used as a hybridization probe for mRNA obtained from elicitor-treated and non-elicitor treated plants. The hybridization mix is subjected to RNase, and those antisense RNA transcripts that were protected from degradation by elicitor-treated RNA but were not protected from degradation by RNA from non-elicitor treated plants are identified. The promoter from the genomic clone which yielded the antisense RNA transcript that was protected by only RNA from elicitor-treated plants is inducible with elicitor.

In another aspect, the invention discloses an amplification method for identifying agrichemicals which can induce expression of a plant gene. According to this aspect of the invention, RNA from plant material not exposed to a putative inducer is isolated and from within this isolated RNA, an RNA encoded by a gene of interest is identified by hybridization to a probe. The RNA from this identified gene is amplified using the self-sustained sequence replication or 3SR™ (SIBIA, La Jolla, CA 92037-4641) technique for amplification of specific RNA sequences and primers specific for the inducible gene transcript of interest. *See* Guatelli, *et al.,* 1990. *Proc. Natl. Acad. Sci USA.*

87:1874-1878. The unexposed plant material from which the first RNA was obtained is then exposed to a putative chemical inducer. RNA encoded by the gene of interest is then identified and amplified using 3SR. The amplification products from unexposed and exposed plant material are compared. If the level of expected product in the amplification of RNA from exposed plant material is higher than in the amplification of RNA from unexposed plant material, it can be concluded that this agrichemical(s) can induce expression of the gene of interest.

With regard to the amplification method, its sensitivity has two significant advantages. Firstly, very small samples can be analyzed, thus for the first time permitting use in agrichemical screens of plants such as *Arabidopsis* and cells in tissue culture. Secondly, some chemicals may have a very small but very significant effect on plant genes, *e.g.*, plant defense genes, such that the response may be difficult to detect with methods less sensitive than 3SR. The 3SR amplification method of the invention makes it possible for the first time to identify these chemical elicitors.

In yet another aspect, the invention discloses a novel method for identifying organisms which can induce plant defense genes. According to this method, a transgenic plant containing at least one chimeric DNA sequence comprised of an inducible plant defense gene promoter operatively linked to at least one reporter structural gene is exposed to a potentially inducing organism. The organism can be living, or can be dead or otherwise disabled. By monitoring expression of the reporter gene it is possible to conclude which organisms induce transcription of the reporter gene, and thus are candidates for use as "inducers" or plant vaccines, or are potentially pathogenic to plants.

The invention will now be described in greater detail in the following examples, which are presented solely for purposes of illustration and not of limitation.

EXPERIMENTAL

POTATO

EXAMPLE I: POTATO

ISOLATION of ARACHIDONIC ACID-INDUCED cDNAs from POTATO

A. Construction of cDNA Library

Potato tubers (*Solanum tuberosum* cv. Desirée) were obtained from the United States Dept. of Agriculture. Tubers were washed with deionized water to remove soil, then soaked in 95% ethanol for one min and rinsed three times with sterile distilled water. Tubers were peeled and surface sterilized in 10% Purex™ (commercial bleach) containing two drops TWEEN 20™ per 100 ml solution for 15 min and rinsed three times with sterile distilled water. Cylinders of tissue were excised with a cork borer (approximately 8 mm in diameter) inserted through the long axis of perimedullary tissue of the tuber and sliced into discs of approximately 3 mm thickness. Tuber discs were transferred to potato callus initiation medium. The potato callus initiation medium contained Murashige and Skoog basal medium (MS medium, Murashige and Skoog, 1962, *Physiol. Plant.* 15:493-497) supplemented with 10 mg/l 2,4-Dichlorophenoxyacetic acid.

Initiated calli were multiplied and maintained on LS2T medium.

Potato Table I: LS2T Medium

Constituent	mg/l
Murashige and Skoog inorganic salts	
Thiamine•HCl	0.5
Pyridoxine•HCl	0.5
Nicotinic acid	5.0
Glycine	2.0
Biotin	0.05
Folic acid	0.5
trans-Zeatin	0.1
2,4-Dichlorophenoxyacetic acid	2.0
Myo-inositol	100.0

Sucrose	30,000.0
Tissue culture agar	8 g/l
pH 5.8	

Potato cell suspension cultures were initiated from potato callus cultures. The cultures were maintained and proliferated on LS2T liquid (or suspension) medium and incubated at 27°C in the dark on a gyratory shaker at a speed of 125 rpm.

Desirée suspension cultures were subcultured weekly. At each subculture, five grams fresh weight of cells were transferred to 100 ml fresh LS2T liquid medium. A five-fold increase in growth was observed in a seven-day growth period. Three hours prior to isolation of RNA, the culture was treated with arachidonic acid (Sigma, St. Louis, MO) to a final concentration of 0.1 mM. Arachidonic acid is a fatty acid normally found in fungal cell walls and induces at least some PAL gene expression (Fritzemeier *et al.*, 1987, *Plant Physiol.* 85:34-41).

After induction, total RNA was isolated from the cultured cells following a modification of the procedure described in Haffner *et al.*, 1978, *Can. J. Biochem.* 56:729-733.

With the modified procedure, the RNA was isolated as follows:

1. Measure out 3-5 g of tissue. Grind into a fine powder using a mortar and pestle (pre-cooled with liquid N_2).
2. Add tissue to pre-chilled 10 ml equilibrated phenol + 5 ml 0.1 M Tris-Cl (pH 9). Homogenize using vortex. All the following steps should be performed at 4°C.
3. Centrifuge 10 minutes at 3,000 x g. Transfer aqueous phase to a 50 ml tube.
4. Back extract phenol. Add 5 ml 0.1 M Tris-Cl (pH 9), mix and re-centrifuge.
5. Combine aqueous phases, add one volume chloroform, mix and re-centrifuge. Remove lower, chloroform phase. Repeat.

6. Transfer aqueous phase to another tube and add 0.1 volume 3 M sodium acetate (pH 5.2) + 2.5 volumes ethanol. Keep at -20°C for 2 hours (minimum).
7. Pellet RNA 30 minutes at 10,000 x g in 30 ml Corex tubes.
8. Resuspend pellet in 6 ml H_2O. When dissolved, add 2.0 ml 8 M LiCl. Mix well. Keep at +4°C overnight.
9. Centrifuge LiCl-precipitated RNA 30 minutes at 10,000 x g. Wash pellet with 10 ml 70% ethanol and re-centrifuge. Decant and remove liquid.
10. Resuspend pellet in 5 ml H_2O.
11. Add 0.5 ml 3 M sodium acetate (pH 5.2) plus 13 ml ethanol. Keep at -20°C for 2 hours (minimum).
12. Centrifuge RNA for 30 minutes at 10,000 x g. Wash with 5 ml 70% ethanol, re-spin, and dry.
13. Resuspend pellet in 1 ml H_2O. Store at -70°C.

Poly A^+ RNA was isolated from 400 μg total RNA using the mRNA Purification Kit (Pharmacia, Piscataway, NJ; catalog #27-9258) and following the manufacturer's instructions. Approximately 3.1 μg poly A^+ was recovered, and used for cDNA synthesis following the BRL cDNA synthesis system protocol (BRL, Bethesda, MD; catalog #8269 SA). The cDNA was treated for 30 min with 9 units T4 DNA polymerase (BRL), following which time a 50-fold molar excess of *Eco*RI adapters (Pharmacia) were ligated to the cDNA (overnight at 15°C). The cDNA was separated from the excess adapters through a 1 ml G50 spin column (Sephadex G50 in a 1 ml syringe, according to Maniatis *et al.*, *in* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982) and the purified cDNA was ligated to *Eco*RI-digested λgt10 arms (Promega, Madison, WI) according to manufacturer's instructions. The entire ligation mixture was packaged using Gigapack™11 Gold Packaging Extract (Stratagene, La Jolla, CA) following manufacturer's instructions. The cDNA library was titered using

E. coli strain C600 (Promega, Madison, WI). The titer of the library was determined to be 2.6 X 10^7 plaque forming units/ml.

B. Library Screening

The library was screened with a 0.9 kb fragment of plasmid pCP63.15, a pUC19-based plasmid comprising a cDNA encoding a portion of the potato PAL exon II gene sequence (Fritzemeier *et al.*, 1987, *Plant Physiol.* 85:34-41). The screening conditions were as follows:

Hybridization:	42°C, 35% Formamide, 5X Denhardts, 5X SSC, 0.2% SDS, 200 μg/ml salmon sperm DNA.
Wash:	2X SSC, 0.1% SDS, repeated a total of four times, at one hour each, at 50°C.

The screening and five rounds of plaque purification identified three putative PAL-encoding cDNAs, which were called λpPAL-3, λpPAL-21, and λpPAL-25.

C. Subcloning and Characterization

All ligations were performed with a ratio of insert to vector of 5:1.

Each of the three lambda clones, λpPAL-3, λpPAL-21, and λpPAL-25, was digested with *Eco*RI, which released the 450, 500, and 250 bp inserts, respectively. After gel purification each insert was ligated to *Eco*RI-digested pUC119 (Vieira, J., and Messing, J., 1987, *In* Methods in Enzymology (R. Wu and L. Grossman, Eds.) Vol. 153 pp. 3-11, Academic Press, New York) and the ligations were separately transformed into DH5α cells. Amp^R colonies were selected. Colonies carrying correct plasmid released a 450, 500, or 250 bp fragment, respectively, upon digestion of plasmid DNA with *Eco*RI.

The *Eco*RI inserts were sequenced using the Sequenase (U.S. Biochemical, Cleveland, OH) protocol for double-stranded DNA, and the nucleotide sequence of each was compared to the nucleotide sequence of the bean gPAL-2 gene (Cramer *et al.*, 1989, *Plant Mol. Biol.* 12:367-383). The similarity was as follows:

Potato Clone #	Homology to gPAL-2

3	55%
21	62%
25	no significant homology

EXAMPLE II: POTATO

ISOLATION of GENOMIC CLONES CORRESPONDING to ARACHIDONIC ACID-INDUCED cDNA from POTATO

A. Construction of Genomic Library

A genomic library was constructed from total genomic DNA isolated from young leaves of *Solanum tuberosum* cv. Desirée. DNA isolation was by the procedure of Bendich, 1988, in Plant Molecular Biology Manual, Kluwer Academic Publishers, Section A6, p. 1-10. The genomic DNA was partially digested with *Sau*3AI, resulting in fragments of between 9-23 kb in size. The *Sau*3AI ends were partially filled in using dATP and dGTP. Approximately 0.43 μg of fragment were ligated with 1 μg of *Xho*I-digested plasmid λFIXII™ (Stratagene), which had the overhang partially filled in using dTTP and dCTP. The ligation reaction was packaged using the Gigapack™11 Gold packaging mix (Stratagene), following manufacturer's directions.

Four libraries were constructed; three contained approximately 3 x 10^5 clones and the fourth had 7 x 10^5 clones.

B. Library Screening

All four libraries were screened under conditions of high stringency using an equal mixture of isolated fragments from the *Solanum tuberosum* cv. Desirée cDNA clones pPAL-3 (450 bp *Eco*RI fragment) and pPAL-21 (500 bp *Eco*RI fragment) (*see* **Potato Example I.B.**) as probes. The screening conditions were as follows:

Hybridization:	42°C, 50% Formamide, 5X Denhardts, 5X SSC, 0.2% SDS, 200 μg/ml salmon sperm DNA.
Wash:	0.2X SSC, 0.1% SDS, for a total of three washes at 45°C, 15 min each wash; and 0.2X SSC, 0.1% SDS, for one wash at 65°C for 15 min.

A total of 43 putative PAL-encoding genomic DNA clones were identified from this screening. Twelve clones were chosen and subjected to five rounds of plaque purification. Nine clones were selected for further characterization. The selected clones referred to herein are called λpPAL-1 through λpPAL-8.

C. Clone Analysis

Analysis of restriction enzyme digests of these nine clones revealed that λpPAL-6 and λpPAL-7 were overlapping clones with several restriction enzyme fragments in common, and that the remaining seven clones were unique (**Potato Figure 1A**). (A map for λpPAL-2 is shown in **Figure 1B**). A map for λpPAL-5 is not shown because no sequence similarity to PAL has been found in this insert.

Through restriction and hybridization analysis (using the inserts from pPAL-3 and pPAL-21 as probes) the open boxed segments of the genomic clones shown in **Potato Figure 1A** were determined to be the portion of the insert that hybridized to the probe(s). Thus, the boxed segments were excised by digestion with the appropriate restriction enzymes (*see* **Potato Figure 1A**) and ligated into appropriately digested plasmid pGEM-7Z (-) (Promega, Madison, WI) to yield the following subclone plasmids:

Genomic Clone #	**Insert (5'-3')**	**Subclone plasmids**
λpPAL-1	*Bam*HI/*Sst*I	pPAL-1
λpPAL-2	*Eco*RI/*Eco*RI	pPAL-2
λpPAL-3	*Eco*RI/*Eco*RI	pPAL-3
λpPAL-4	*Eco*RI/*Bgl*II	pPAL-4
λpPAL-6	*Hin*dIII/*Eco*RI	pPAL-6
λpPAL-7	*Bam*HI/*Eco*RI	pPAL-7
λpPAL-8	*Eco*RI/*Eco*RI	pPAL-8

The insert DNAs were sequenced according to the Sequenase protocol (U.S. Biochemical Corporation) for double-stranded dideoxynucleotide, and the sequences were analyzed using the University of Wisconsin Genetics (UWG) comparison program, FASTA. The partial sequences of the genomic clones, except λpPAL-5, showed identity to a PAL

cDNA clone isolated from *Solanum tuberosum* cv. Datura. In addition, partial sequences of pPAL-2, pPAL-3, pPAL-4, and pPAL-8 show greater than 90% sequence similarity to each other; the partial sequences of pPAL-6 and pPAL-7 are identical; and the partial sequence of pPAL-1 showed 62% sequence similarity to pPAL-6 and pPAL-7.

These results suggested that the genes corresponding to λpPAL-2, λpPAL-3, λpPAL-4, and λpPAL-8 are members of the same PAL gene subfamily, that λpPAL-6 and λpPAL-7 are genomic clones of the same PAL gene, and that λpPAL-1 contains a PAL gene that is different from the PAL gene contained in λpPAL-6.

EXAMPLE III: POTATO

RNASE PROTECTION ASSAY

RNase protection assays (Winter *et al.*, 1985, *Proc. Natl. Acad. Sci.* 82:7575-7579) are based on the different susceptibility of single-stranded RNA and RNA-RNA hybrids to degradation by the single-stranded RNA-specific enzyme RNase. In these assays, total RNA is hybridized to a radioactively-labeled antisense probe complementary to the transcript of interest, followed by RNase-mediated degradation of all single-stranded RNA. If the transcript of interest is present in total RNA, then a double stranded RNA-RNA hybrid containing no mismatches will have formed and will be unaffected by the RNase treatment. This product can be identified by size following gel electrophoresis.

To determine if the genes from which the genomic subclones described in **Potato Example II.C.** were derived are induced by arachidonic acid, RNase protection assays were performed in which labeled antisense transcripts generated from each subclone were hybridized to total RNA from arachidonic acid-treated and untreated potato (*Solanum tuberosum* cv. Desirée) suspension culture cells. The hybridization mixture was then treated with RNase and analyzed by electrophoresis. Protection of a labeled antisense probe from degradation in the presence of RNA from arachidonic acid-treated cells but not in the presence of RNA from untreated cells would indicate that expression of the corresponding gene is induced by arachidonic acid.

Therefore, this assay method enabled a determination of whether the genes corresponding to the genomic clones were regulated by arachidonic acid-inducible promoters.

A. Protocol

For the RNase protection assays described herein, the transcripts of interest were defined by the genomic subclone inserts as follows:

Genomic Subclone	Insert Used	Subclone for RNase exp.
λpPAL-1	1250 bp *Bam*HI/*Sst*I	pPAL-1*
λpPAL-2	271 bp *Eco*RI/*Pst*I	pPAL-2EP**
λpPAL-3	276 bp *Eco*RI/*Pst*I	pPAL-3EP**
λpPAL-4	276 bp *Eco*RI/*Pst*I	pPAL-4EP*
λpPAL-8	278 bp *Eco*RI/*Pst*I	pPAL-8EP**
λpPAL-6	500 bp *Hin*dIII/*Sst*I	pPAL-6HS**

* The antisense transcript is transcribed using the SP6 promoter and the sense transcript is transcribed using the T7 promoter.

** The antisense transcript is transcribed using the T7 promoter and the sense transcript is transcribed using the SP6 promoter.

The noted inserts were ligated into appropriately digested pGEM-7Z(-), except for the 500 bp insert of λpPAL-6 which was ligated into pGEM-11Z(+) (Promega). The polylinker regions in both these plasmids are flanked by the SP6 and T7 RNA polymerase promoters making it possible to generate ^{32}P-labeled antisense RNA transcripts of the inserts using SP6 or T7 RNA polymerase. The choice of which polymerase to use depends upon the orientation of the gene fragment in the polylinker.

^{32}P-labeled antisense RNA transcripts were synthesized *in vitro* from the subclones pPAL-1, pPAL-2EP, pPAL-3EP, pPAL-4EP, pPAL-8EP, and pPAL-6HS using the Riboprobe Gemini System II (Promega) and following manufacturer's instructions. The RNAs were then used as probes in RNase protection assays of total RNA from *Solanum tuberosum* cv. Desirée suspension culture cells. Suspension cells were prepared as described in **Potato Example I.A.** and treated for three hours with freshly prepared 0.1 mM

arachidonic acid (induced) or water (uninduced) prior to isolation of total RNA from the cells (RNA was isolated as described in **Potato Example I.A.**). The isolated RNAs were separately hybridized overnight at 45°C to each of the six different antisense probes. The hybridization conditions were as follows: 40 mM Pipes, pH 6.4, 1 mM EDTA, 400 mM NaCl, 50% Formamide.

The hybridization mixtures were then digested with a mixture of RNase A (40 μg/ml; Sigma, St. Louis, MO) and RNase T1 (2 U/ml; BRL, Bethesda, MD) at 34°C, and analyzed on a 6% polyacrylamide, 8M urea gel.

To provide positive controls in each of these experiments, sense RNA was transcribed *in vitro* from subclones pPAL-1, pPAL-2EP, pPAL-3EP, pPAL-4EP, pPAL-8EP, and pPAL-6HS and hybridized with the corresponding labeled antisense RNA. These hybrids should be a perfect match.

B. Results

As expected, the antisense RNA transcripts were protected from RNase-mediated degradation when hybridized with sense RNA synthesized from the same subclone, but were completely degraded in the absence of sense RNA.

Antisense transcripts derived from pPAL2-EP, pPAL3-EP, pPAL-4EP, pPAL-8EP, and pPAL-6HS each were protected from degradation by RNA prepared from *Solanum tuberosum* cv. Desireé cells induced with freshly prepared arachidonic acid. This result confirmed that the promoter associated with the gene(s) to which these clones correspond is inducible with arachidonic acid.

Some protection also was observed with RNA prepared from cells which had not been induced, although it was much less than that observed with RNA from induced cells. This result was not surprising as significant background levels of PAL gene expression were consistently observed in these cells.

The probe derived from pPAL-1 was protected equally well from degradation by RNA from both induced and uninduced cells. This result suggested that the gene corresponding to λpPAL-1 is not an arachidonic-acid inducible PAL gene.

EXAMPLE IV: POTATO

ISOLATION of POTATO PAL PROMOTER

A. Cloning

An approximately 6 kb *Sst*I-*Bam*HI fragment from λpPAL-2 was gel purified and subcloned into pGEM11Z(+) (Promega) using standard DNA cloning procedures (Maniatis *et al.*, *supra*). The ligation mixture was transformed into DH5α and Amp^R colonies were selected. A correct construct was confirmed by the presence of 6 and 3.2 kb bands in *Sst*I-*Bam*HI-digested DNA and was called pD2.2.

A 1.4 kb *Kpn*I-*Sst*I fragment and a 3.3 kb *Sst*I-*Eco*RI fragment were gel purified from λpPAL-7. Equal molar ratios of these fragments were mixed and subcloned into pUC119 previously digested with both *Kpn*I and *Eco*RI. The ligation reaction was transformed into DH5α cells and Amp^R colonies were selected. Correct construct was confirmed by the presence of 4.7 and 3.2 kb bands upon double digestion of plasmid DNA with *Eco*RI and *Kpn*I, and was called 7P.

B. Characterization

The complete promoter-containing region and a portion of the coding region of λpPAL-7, and the majority of the promoter-containing region and a portion of the coding region of λpPAL-2 have been sequenced. The two PAL coding regions share 80% sequence similarity at the nucleotide level.

EXAMPLE V: POTATO

ARACHIDONIC-INDUCED GENE EXPRESSION

A. Construction of pUC-GUS.1, pUC-GUS.2, pUC-GUS.3

Promoter-less GUS (β-glucuronidase) gene cassettes are available as *Hin*dIII-*Eco*RI inserts in plasmids pBI101, pBI101.2, and pBI101.3 (Clontech, Palo Alto, CA). The GUS cassettes in plasmids pBI101.2 and pBI101.3 are identical to the one in pBI101 except their reading frames are shifted one and two nucleotides, respectively, relative to the polylinker. As a result, a promoter fragment and a portion of the coding region can be inserted in all three reading frames upstream of the GUS gene creating both translational and transcriptional fusion constructs.

The GUS-encoding insert was removed from each plasmid and each insert was separately ligated with *Hin*dIII-*Eco*RI-digested pUC119. The ligation was transformed into DH5α cells, and Amp^R colonies were selected. Correct plasmids demonstrated bands of 2.2 and 3.2 kb in size upon digestion with *Eco*RI and *Hin*dIII and were called pUC-GUS.1, pUC-GUS.2, and pUC-GUS.3, respectively.

B. Construction of PAL-GUS Fusion Vectors

1. Promoter λpPAL-2

Plasmid pD2.2 (**Potato Example IX.A.**) was digested with *Hae*III, and the insert was separately ligated with *Sma*I-digested pUC-GUS.1, pUC-GUS.2, and pUC-GUS.3. After transformation into DH5α cells, and selection of Amp^R colonies, correct plasmids were identified by release of a 1.8 kb band upon digestion with *Hin*dIII. Correct plasmids were called pPAL2.1, pPAL2.2, and pPAL2.3.

Plasmids pPAL2.1, 2.2, and 2.3 were digested with *Xba*I and *Sst*I and each insert was ligated to *Xba*I- and *Sst*I-digested pBI101. Plasmid pBI101 (Clontech, Palo Alto, CA) is a broad host range plasmid used in the binary vector, plant transformation system of *Agrobacterium tumefaciens*. The ligations were transformed into DH5α cells and Kan^R colonies were selected. Correct plasmids were identified by release of a 6 kb fragment upon digestion with *Xba*I and *Sst*I, and were called pBIN-2.1, pBIN-2.2, and pBIN-2.3, respectively.

2. Promoter λpPAL-7

Plasmid 7P was digested with *Pst*I and *Eco*RV and the insert was ligated with *Pst*I- and *Sma*I-digested pUC-GUS.1, pUC-GUS.2, and pUC-GUS.3. After transformation into DH5α cells and selection of Amp^R colonies, correct plasmids were identified by release of a 4.7 kb band upon digestion with *Eco*RI and *Bam*HI. Correct plasmids were called pGUS7.1, pGUS7.2, and pGUS7.3, respectively.

Plasmid pGUS7.1, 7.2, and 7.3 were digested with *Hin*dIII and *Eco*RI, and each insert was ligated to *Hin*dIII- and *Eco*RI-digested pBIN19. Plasmid pBIN19 is a broad host range plasmid used in the binary vector

Agrobacterium tumefaciens transformation system and is available from Clontech. The ligations were transformed into TB-1 cells and Kan^R colonies were selected. Correct plasmids were identified by release of a 6 kb fragment upon digestion with *Hin*dIII and *Eco*RI and were called pBIN7.1, pBIN-7.2, and pBIN-7.3, respectively.

Prior to transformation of potato, plasmids pBIN2.1, 2.2, 2.3, 7.1, 7.2 and 7.3 were transferred from the *E. coli* host to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating using pRK2073 as the helper strain (Corbin, D. *et al.*, 1982, *J. Bacteriol.* 149:221-228).

C. Transformation of Potato

The potato tubers used in this experiment were obtained from potato plants of *Solanum tuberosum* cv. Desirée. Tubers that had been stored in the dark at 4°C for one week were washed and rinsed with deionized water to remove soil, surface sterilized one minute in 95% ethanol and rinsed in sterile distilled water. The tubers were peeled and disinfected for 15 minutes in 10% Purex™ (commercial bleach) containing two drops of Tween 20™ per 100 ml solution followed by five rinses with sterile distilled water. The proximal and distal quarter portions of the tubers were discarded. The sterilized potato tubers were immersed in MS liquid medium without hormones (Murashige and Skoog, 1962, *Physiol. Plant* 15:473-496) for 20 min prior to removal of the discs.

The explants were floated in 20 mls of MS liquid medium without hormones containing an overnight culture of separate *Agrobacterium tumefaciens* LBA 4404 (Clontech, Palo Alto, CA) strains harboring plasmids pBIN2.1, 2.2, 2.3; and pBIN7.1, 7.2, 7.3 (**Potato Example IV.B.**). The *Agrobacterium* strains were pre-induced with 50 μM acetosyringone. The tissue and *Agrobacterium* were incubated at room temperature on a gyratory shaker with gentle shaking (at approximately 60 rpm). After 20 minutes, the explants were blotted on sterile Whatman paper No. 1 and transferred to incubation media which were two-day-preconditioned tobacco feeder plates. Feeder plates were prepared according to the method of Horsch and Jones (*In Vitro*, 1980, 16:103-108) with the following modification: cells from six-day-old

suspension culture were filtered through a sterile 30-mesh sieve, collected on two layers of sterile kimwipes and freed of excess medium through a funnel. Cells were then resuspended in fresh MM medium to a final density of 0.3 g fresh weight per ml. MM medium contained MS basal medium supplemented with 0.5 mg/l 2,4-D and 0.5 mg/l BA. The suspension was stirred and 1.5 ml aliquots were pipetted onto plates containing two kinds of shoot regeneration media, 3C52R medium (Steerman and Bevan, 1988, (*Plant Cell Reports* 7:13-16)) and the medium defined in Jarrett *et al.* (*Physiol. Plant.*, 1980, 49:177-184).

After two days, infected tuber discs were transferred to selection media containing cefotaxime (500 μg/ml) and kanamycin (100 μg/ml). The composition of the media was identical to incubation media, but there was no feeder layer. Tissues were transferred to fresh selection media at two-week intervals, and the cefotaxime concentration was reduced to 250 μg/ml after four weeks in culture. Potato shoots were regenerated after three weeks in culture. When regenerated potato shoots reached 3-5 mm in size, they were excised from tuber discs and grown on the selection media containing 250 μg/ml cefotaxime and 100 μg/ml kanamycin.

When putative transgenic potato shoots reached 2 cm in size, they were transferred to rooting media containing 250 μg/ml cefotaxime and 100 μg/ml kanamycin. The rooting media were identical to shoot regeneration media except plant hormones were not added. When the shoots had rooted, the resulting plantlets were transplanted from culture into soil and grown in a plant growth chamber. At desired times, the transgenic potato plants are assayed for GUS activity.

The cultures were incubated at 27°C with 16 hours of light under 4000 lux light intensity throughout the experiments.

EXAMPLE V: POTATO
USE OF TRANSGENIC POTATO PLANTS
AS AN AGRICHEMICAL SCREEN

Transgenic potato plants containing PAL-GUS fusion constructs are clonally propagated from tubers derived from transgenic plants shown to

contain stably-integrated PAL-GUS constructs which are induced, in this example, by arachidonic acid. These transgenic plants are maintained under standard greenhouse conditions.

For use in agrichemical screening assays, plants are grown to a size and/or stage of development which is both manageable under greenhouse conditions and susceptible to a pathogen of interest, *e.g., Phythophthora infestans*. The plants are treated either by foliage spray or soil immersion with a range of concentrations of the unknown chemical (elicitors). In parallel, additional plants are exposed to water as a negative control or to a known elicitor as a positive control. Six to twenty four hours following treatment, leaf tissue is removed and assayed for GUS activity using the fluorometric assay of Jefferson *et al.* (*Plant Mol. Biol. Rep.*, 1987, 5:387-405). A positive result is indicated by levels of GUS activity significantly higher than those observed with the negative control. A positive result is indicative of, *e.g.*, identifies, chemicals which are capable of inducing potato PAL promoter(s).

RICE

EXAMPLE VI: RICE

ISOLATION of PAL PROMOTERS from RICE GENOMIC DNA

A. Screening Rice Genomic Library

A rice (*Oryza sativa*) genomic library (Clontech, Palo Alto, CA; average insert size of 15 kb in λEMBL3) was screened with elicitor-inducible bean PAL1 exonII cDNAs to identify clones encoding rice PAL genes. The cDNAs encoding PAL1 exonII sequences were pPAL1-B6 and pSPP1 (both obtained from C. Lamb, The Salk Institute, La Jolla, CA; no published reference). The probe was an equal mixture of the 800 bp insert of pPAL1-B6 and the 500 bp insert of pSPP1. The screening conditions were as follows:

Hybridization:	42°C, 35% Formamide, 5X SSC, 5X Denhardts, 0.2% SDS, 200 μg/ml salmon sperm DNA.
Wash:	2X SSC, 0.1% SDS, repeated a total of four times, one hour each, at 50°C.

Five clones comprising putative rice PAL sequences were selected following six rounds of plaque purification. Clones λrPAL-2, -4, and -10 gave strong signals when hybridized to the probe, whereas clones λrPAL-8 and -12 hybridized weakly.

B. Clone Characterization

1. Restriction Mapping

The five clones were characterized by restriction enzyme analysis. Clones λrPAL-8 and -12 were identical; clones λrPAL-4 and -10 had several fragments in common; and clone λrPAL-2 was unique. Partial restriction enzyme maps of inserts from clones λrPAL-2, -4, and -10 are shown in **Rice Figure 2.**

2. DNA Sequencing

Through restriction enzyme mapping and hybridization analysis using the bean PAL1 exon II DNA fragments as probe, the portions of clones λrPAL-2, -4, -8, and -10, which contained PAL-hybridizing regions were determined. These portions are shown in **Rice Figure 2** by a stippled box for λrPAL-2, and cross-hatched boxes for λrPAL-4 and -10. The boxed fragments were excised from the λrPAL clones and ligated to appropriately digested pUC119 (Vieira and Messing, 1987, *Meth. Enzym.* 153:3-11). After transforming the ligation reaction into DH5α cells and selecting for Amp^R colonies, correct plasmids were identified by release of an appropriately sized fragment (*i.e.*, insert size) upon digestion with the appropriate enzymes. The stippled region of λrPAL-2 was in p6, and the cross-hatched regions of λrPAL-4 and -10 were in p296 and p4410, respectively.

The insert DNAs were sequenced according to the Sequenase protocol (U.S Biochemical Corporation, Cleveland, OH) for double-stranded dideoxynucleotide sequencing. The partial sequence of the PAL region of λrPAL-2 was found to have 84.3% identity to the published sequence of a rice (*Oryza sativa* cv. Nipponbare) genomic PAL gene (Minami *et al.*, 1989, *Eur. J. Biochem.* 185:19-25); the partial sequences of the fragments from λrPAL-4 and λrPAL-10 were the same and showed 79.6% identity to the rice PAL genomic sequence; and the partial sequence of the λrPAL-8 fragment has 53.8%

identity to the rice PAL genomic sequence. These data suggested that clones λrPAL-2, λrPAL-4, and λrPAL-10 contain portions of the rice PAL genes, and that λrPAL-8 may or may not contain a rice PAL gene.

The orientation of the PAL genes within clones λrPAL-2, -4, and -10 was determined from the sequence data. Based on the size and orientation of the inserts compared to the published sequence of a rice PAL gene (Minami *et al.*, 1989, *supra*) it appeared likely that all three clones contained rice PAL promoters. To localize the promoter regions of these clones, fragments were further subcloned and sequenced.

For clone λrPAL-2, both the *Hin*dIII-*Eco*RI and *Eco*RI-*Hin*dIII fragments (**Rice Figure 2**), striped boxes were separately subcloned into pUC119. Because the region of similarity between the λrPAL-4 and λrPAL-10 clones and the 5′ end of the published rice PAL genomic sequence lies at the right end of the *Sal*I fragment (cross-hatched boxes in **Rice Figure 2**), the *Sal*I fragment (black box) from λrPAL-10 was subcloned into pUC119 and sequenced. The promoter fragment from λrPAL-4 was subcloned as a 3 kb *Cla*I-*Sst*I fragment into *Acc*I-*Sst*I digested pGEM5Z (Promega, Madison, WI). This 3 kb fragment contains about 300 bp of the right arm of EMBL3, the 1800 bp *Sal*I fragment (black box in **Rice Figure 2**) and a portion of the 1850 *Sal*I fragment (hatched box).

The composite sequence of λrPAL-4 and -10 is shown as **SEQ ID NO. 9** (λrPAL-4). The sequence of the promoter region and a portion of the coding region of λrPAL-2 is shown in **SEQ. ID NO. 8** (λrPAL-2).

The sequencing data showed that the promoter regions of λrPAL-4 and λrPAL-10, as well as a portion of their coding regions, are identical, except that λrPAL-4 contains an additional 850 bp of upstream sequence compared to λrPAL-10. Comparison between the published rice (*Oryza sativa* cv. Nipponbare) PAL genomic sequence (Minami *et al.*, 1989, *supra*) and the sequences of the subclones of λrPAL-4 and λrPAL-10 showed that the translational start (ATG) of the published sequence is located five base pairs upstream of the *Sal*I restriction site (position 1724 in **SEQ ID NO. 9**) in

λrPAL-4 and λrPAL-10. Therefore, it is likely that the translational start site of the PAL gene encoded by λrPAL-4 and λrPAL-10 is near this *Sal*I site.

A plant translational start consensus sequence (GNNATGG) is present at position 1704 of the composite sequence in (SEQ ID NO. 9) in λrPAL-4 and λrPAL-10. Computer-generated translation of the nucleotide sequence starting with the ATG at position 1702 and extending to position 1828 yielded protein sequence which shows 78% similarity to the amino acid sequence predicted from the published rice PAL gene sequence (Minami *et al.*, 1989, *supra*) and 73% similarity to the amino acid sequence deduced from bean gPAL-2 (Cramer *et al.*, 1989, *Plant Mol. Biol.* 12:367-383).

The putative translational start of λrPAL-2 is located at nucleotide position 1873 of SEQ ID NO. 8. Computer generated translation of the nucleotide sequence starting at this position results in protein sequence which shows 80% similarity to the amino acid sequence predicted from the published rice PAL gene sequence (Minami *et al.*, 1989, *supra*), and 51% similarity to the amino acid sequence deduced from λrPAL4 and λrPAL-10.

EXAMPLE VII: RICE

CONSTRUCTION of RICE PAL-GUS CONSTRUCTS

A. Construction of GUS Marker-Hygromycin Selectable Base Expression Vectors

Promoter-less GUS (β-glucuronidase) gene cassettes are available as *Hin*dIII-*Eco*RI inserts in plasmids pBI101, pBI101.2, and pBI101.3 (Clontech, Palo Alto, CA). The GUS cassettes in plasmids pBI101.2 and pBI101.3 are identical to the one in pBI101 except their reading frames are shifted one and two nucleotides, respectively, relative to the polylinker. As a result, inserting a promoter fragment and a portion of the gene coding region into each vector creates both translational and transcriptional fusion constructs in all three reading frames upstream of the GUS gene.

All ligations were performed with an insert to vector molar ratio of 5:1. Thus, each of the three plasmids, pBI101, pBI101.2, and pBI101.3 was digested with *Hin*dIII and *Eco*RI and the GUS-encoding insert was removed and purified on a 1% TBE gel. Each insert was ligated separately with

*Hin*dIII-*Eco*RI-digested pUC119. The ligation was transformed into DH5α cells, and Amp^R colonies were selected. Correct plasmids demonstrated bands of 2.2 and 3.2 kb in size upon digestion with *Eco*RI and *Hin*dIII and were called pUC-GUS.1, pUC-GUS.2, and pUC-GUS.3, respectively.

Plasmid pSV2hyg (obtained from J. Kwoh, Baxter Healthcare, San Diego, CA) containing the *E. coli* hygromycin B gene, was digested with *Hin*dIII and *Bgl*II and the entire digest was treated with T4 DNA polymerase (BRL, Bethesda, MD). The fragment comprising the hygromycin gene was isolated on a 1% TBE gel and ligated to *Pst*I-digested, T4 DNA polymerase-treated pCAMVCN DNA (Pharmacia, Piscathaway, NJ) using established cloning procedures. The ligation reaction was transformed into DH5α cells and Amp^R colonies were selected. The correct plasmid released a 2 kb fragment upon digestion with *Hin*dIII and was called p35S-hyg.

Plasmid p35S-hyg was digested with *Hin*dIII and the 2 kb fragment was purified on a 1% TBE gel. The fragment then was ligated separately into the pUC-GUS vectors (pUC-GUS.1, pUC-GUS.2, and pUC-GUS.3) which had been digested with *Hin*dIII. The ligation reaction was transformed into DH5α cells and Amp^R colonies were selected. The correct plasmids released a 2 kb fragment upon digestion with *Hin*dIII and were called pHyg-GUS.1, pHyg-GUS.2, and pHyg-GUS.3, respectively. In all three plasmids, the direction of transcription of the hygromycin resistance gene was opposite to that of the GUS gene; therefore, any GUS activity detected in plants transformed with these vectors will not be due to read-through transcription from the CaMV35S promoter.

B. Construction of Expression Vectors Having Rice PAL Promoters

1. Clone λrPAL-4

The promoter-carrying fragment from λrPAL-4 was isolated from the *Cla*I-*Sst*I subclone (*see* **Rice Example VI.B.2.**) following digestion of the subclone with *Bss*HII, treatment with Klenow, and digestion with *Pst*I. The approximately 1850 bp resultant fragment was ligated separately into pHyg-GUS.1, pHyg-GUS.2, and pHyg-GUS.3 plasmids (*see* **Rice Example VII.A.**),

previously digested with *Sma*I and *Pst*I. The ligations were transformed into DH5α cells and Amp^R colonies were selected. The correct constructs were determined by linearization of a 7000 bp plasmid upon digestion with *Eco*RI, and were called plasmids rPAL4.1, rPAL4.2, and rPAL4.3, respectively.

2. Promoter from λrPAL-10

The promoter fragments from λrPAL-10 were isolated by the following method. Plasmid pSal1000 (the 800 bp *Sal*I fragment from black boxed portion of λrPAL-10 in **Rice Figure 2**, ligated into pUC119) was digested with *Sal*I and *Pst*I, and the 800 bp fragment was purified on a 1% TBE gel. Plasmid p4410 (*see* **Rice Example VI.B.2.**) was digested with *Bss*HII, treated with Klenow, digested with *Sal*I and *Sst*II (to remove the other *Bss*HII-*Sal*I fragment which would interfere in the ligation). The 800 bp fragment and the entire *Sal*I-*Sst*II digestion were ligated into *Sma*I- and *Pst*I-digested pHyg-GUS.1, pHyg-GUS.2, and pHyg-GUS.3 in three way ligations. The ligations were transformed into DH5α cells and Amp^R colonies were selected. The correct constructs were determined by linearization of an approximately 6200 bp plasmid upon digestion with *Eco*RI, and were called rPAL10.1, rPAL10.2, and rPAL10.3.

3. Promoter from λrPAL-2

The promoter fragment from λrPAL-2 was digested with *Eco*RI, treated with Klenow, and phosphorylated *Bam*HI linkers (New England Biolabs, Inc., Beverly, MA) were added. The DNA then was digested with *Not*I, treated with Klenow, and digested with *Bam*HI. The resultant approximately 2100 bp fragment was ligated separately into *Bam*HI-*Sma*I digested pHyg-GUS.1, pHyg-GUS.2 and pHyg-GUS.3 according to established cloning procedures. The ligations were transformed into DH5α cells and the correct constructs were determined by linearization of the approximately 7 kb plasmid upon digestion with *Bam*HI. Correct plasmids were called rPAL2.1, rPAL2.2, and rPAL2.3

EXAMPLE VIII: RICE

previously digested with *Sma*I and *Pst*I. The ligations were transformed into DH5α cells and Amp^R colonies were selected. The correct constructs were determined by linearization of a 7000 bp plasmid upon digestion with *Eco*RI, and were called plasmids rPAL4.1, rPAL4.2, and rPAL4.3, respectively.

2. Promoter from λrPAL-10

The promoter fragments from λrPAL-10 were isolated by the following method. Plasmid pSal1000 (the 800 bp *Sal*I fragment from black boxed portion of λrPAL-10 in **Rice Figure 2**, ligated into pUC119) was digested with *Sal*I and *Pst*I, and the 800 bp fragment was purified on a 1% TBE gel. Plasmid p4410 (*see* **Rice Example VI.B.2.**) was digested with *Bss*HII, treated with Klenow, digested with *Sal*I and *Sst*II (to remove the other *Bss*HII-*Sal*I fragment which would interfere in the ligation). The 800 bp fragment and the entire *Sal*I-*Sst*II digestion were ligated into *Sma*I- and *Pst*I-digested pHyg-GUS.1, pHyg-GUS.2, and pHyg-GUS.3 in three way ligations. The ligations were transformed into DH5α cells and Amp^R colonies were selected. The correct constructs were determined by linearization of an approximately 6200 bp plasmid upon digestion with *Eco*RI, and were called rPAL10.1, rPAL10.2, and rPAL10.3.

3. Promoter from λrPAL-2

The promoter fragment from λrPAL-2 was digested with *Eco*RI, treated with Klenow, and phosphorylated *Bam*HI linkers (New England Biolabs, Inc., Beverly, MA) were added. The DNA then was digested with *Not*I, treated with Klenow, and digested with *Bam*HI. The resultant approximately 2100 bp fragment was ligated separately into *Bam*HI-*Sma*I digested pHyg-GUS.1, pHyg-GUS.2 and pHyg-GUS.3 according to established cloning procedures. The ligations were transformed into DH5α cells and the correct constructs were determined by linearization of the approximately 7 kb plasmid upon digestion with *Bam*HI. Correct plasmids were called rPAL2.1, rPAL2.2, and rPAL2.3

EXAMPLE VIII: RICE

Protoplasts were purified by centrifugation through a Percoll Solution step gradient. Protoplasts were resuspended in 6 ml of 70% Percoll Solution (**Rice Table III**), a 50% Percoll Solution (6 ml) was layered on top of the resuspended protoplasts, and 6 ml of a 25% Percoll Solution was layered on top of the 50% Percoll Solution.

Rice Table III: Percoll Solution

(1)	70% Percoll Solution	
	Percoll solution	70.0 ml/100 ml
	Commercial Murashige and Skoog salt base	0.43 g/100 ml
	Sucrose	3.0 g/100 ml
	Mannitol	0.3M
(2)	50% Percoll Solution	
	Percoll solution	50.0 ml/100 ml
	Commercial Murashige and Skoog salt base	0.43 g/100 ml
	Sucrose	3.0 g/100 ml
	Mannitol	0.3M
(3)	25% Percoll Solution	
	Percoll solution	25.0 ml/100 ml
	Commercial Murashige and Skoog salt base	0.43 g/100 ml
	Sucrose	3.0 g/100 ml
	Mannitol	0.3M

The Percoll-protoplast gradient was centrifuged at approximately 297 x g for 15 minutes at room temperature. Protoplasts were collected at the interface of the 25% and 50% Percoll Solutions using a sterile pasteur pipet and transferred to 25 ml of Protoplast Wash Solution. Protoplasts were washed twice by resuspending in 25 ml Protoplast Wash Solution and centrifuging at approximately 147 x g for 10 minutes after each resuspension. The protoplasts were resuspended in Protoplast Wash Solution to a density of approximately 1 x 10^7 protoplasts per ml for transformation experiments.

PEG-mediated transformation was conducted as follows: One-milliliter aliquots of protoplasts were separately mixed with 100 μg/ml plasmid DNA from either rPAL2.2 or rPAL4.3 (*see* **Rice Example VII.B.1. and 3).** An equal volume of polyethylene glycol (PEG 8000, 40% w/v) in medium (Krens' F solution; Krens *et al.*, 1982, *Nature* 296:72-74) was added to the mixtures of protoplasts and plasmid DNAs. The protoplast-PEG mixture was heat shocked at 45°C for five minutes followed by chilling on ice for 20 seconds. The solution was then brought to room temperature followed by incubation at 30°C for 30 minutes. The protoplast-PEG mixture then was diluted with Krens' F solution until the PEG concentration was less than 2% according to the following time schedule as a reference:

0- 2 min	two drops every 30 sec
2- 5 min	five drops every 30 sec
5-10 min	0.5 ml every 30 sec
10-15 min	1 ml every 30 sec
15-30 min	2 ml every 5 min

The protoplasts treated with plasmid DNA were collected by centrifugation at approximately 147 x g for 10 minutes and resuspended in N6 medium **(Rice Table II)** containing 0.3 M mannitol and incubated in the dark at room temperature on a gyratory shaker at ~50 rpm.

Samples of each protoplast mixture were treated with fungal elicitor prepared from the cell walls of *Pyricularia oryzae*. Preparation of fungal elicitor is described in below, in **Rice Table IV.**

Table IV: Rice: Preparation of Fungal Elicitor

Maintenance media:	Corn meal agar (Difco™ 0386-01-3); plate.
Growth media:	Liquid culture.
Corn meal broth:	Blend 50 g corn meal in 800 ml of distilled H_2O; refrigerate mixture overnight; then heat for about 1 h at 60°C; filter

solution; bring solution to 1 liter; autoclave for 20 min.

Double autoclave (120°C/20 min) all media and instruments. Leave plates/liquid media for > 1 week to check for contamination.

To maintain cultures: take 1 x 0.6 mm cork borer disc and place in center of corn meal agar dish. Take the sample from the edge of dark grown mycelia.

To innoculate growth media: take 5 x 0.6 mm cork borer discs from edge of growing mycelia/50 ml media (in 250 ml conical flask).

Maintain at in the dark at 23°C. Harvest mycelia after 1 month.

Preparation of crude elicitor from *Pyricularia oryzae* mycelial walls.

1. Homogenize mycelium in Waring™ blender for 60 sec (5 ml H_20 per gram wet weight of mycelia). Filter homogenate through a coarse scintered glass filter*; retain the residue. (N.B., it is often necessary to clear the glass filter with conc. nitric acid and then rinse well with distilled H_2O, since the pores clog up.)
2. Repeat step 1 three more times*.
3. Homogenize once with a mixture of chloroform: methanol (1:1) (1 g mycelia per 5 ml of fluid); filter*.
4. Homogenize once with acetone (1 g mycelia to 5 ml of acetone); filter.
5. Air dry at room temperature. This treatment leaves mycelial wall fraction.
6. Suspend 5 g of walls in 100 ml of water and autoclave for 1 11/2 hr at 121°C.
7. Autoclaved suspension was filtered through a course scintered glass filter.
8. Concentrate by freeze drying (lyophilization).
9. Resuspend in sterile (approx. 10 ml) H_2O.
10. Do carbohydrate and protein assay.

* This may vary. Do until filtrate is clear, at final acetone stage.

Refs: Ayers, *et al., Plant Physiol.*, 1976, 57:760.

Dixon, *Planta*, 1981, 157:272.

Fungal elicitor at a concentration of approximately 60 or 80 μg/ml was added 22 (80 μg) or 36 (60 μg) hours after transformation, and incubation was continued for an additional 20 hours. Following this 42 or 56 hour incubation, control and treated samples were processed as described below.

1. Transient Expression Assays

After 40 hours of incubation post-transformation, rice protoplasts that had been transformed with the rice PAL-GUS fusion constructs were collected by centrifugation in a microfuge for about 30 seconds. Protoplasts were homogenized in 50 μl GUS extraction buffer (Jefferson, 1987, *Plant Mol. Biol. Rep.* 5:387-405). Another 150 μl of GUS extraction buffer was added, and protoplasts were frozen in liquid nitrogen, then thawed at room temperature. This procedure was repeated, and the protoplast mixture was centrifuged in a microfuge at 4°C for 10 minutes. Protoplast extracts (100 μl) were incubated with 0.6 ml MUG buffer containing 20% methanol according to the procedure of Jefferson (1987, *supra*) to determine GUS activity. Briefly, the assay for GUS activity was a fluorometric assay which measures the production of 4-methyl umbelliferyl from 4-methyl umbelliferyl glucuronide (MUG), a fluorogenic substrate. Protein concentration was determined according to the Bradford Protein Assay using reagents obtained from Bio-Rad.

2. Results

Protoplasts were separately transformed with two rice PAL-GUS fusion constructs, rPAL2.2 and rPAL4.3, each containing a translational fusion between a rice PAL promoter and the GUS gene. The extracts of these protoplasts were assayed for GUS activity. The results of these assays are shown in **Rice Table V.**

Rice Table V: GUS Activity in Rice Protoplasts Transformed with Rice PAL-GUS Fusion Construct rPAL2.2

		GUS Activity (pmol/min/mg)[a]	% Increase with elicitor
Experiment 1:	No DNA control	26.	-
	rPAL 2.2	785.	-
	rPAL 2.2 + elicitor	862.	9.8%
	rPAL 4.3	86.	-
	rPAL 4.3 + elicitor	70	-
Experiment 2:	rPAL 2.2	1,542.	-
	rPAL 2.2 + elicitor	1,707.	10.7%
Experiment 3:	rPAL 2.2	2,464.	-
	rPAL 2.2 + elicitor	2,683.	8.8%
	rPAL 2.2 + elicitor	2,655.	7.7%

[a]These values are an average of data obtained at different time points in the enzyme assay.

a. Background GUS Expression

A higher level of uninduced GUS activity was measured in protoplasts transformed with plasmid DNA, but not induced with elicitor, than in control protoplasts not transformed and not induced. The higher GUS levels are due to a certain level of constitutive and/or induced (induction other than elicitor-mediated induction) GUS expression in the experimental transformed protoplasts and are indicative of successful transfer of the constructs into the protoplasts. The difference in the background GUS levels of protoplasts transformed with rPAL2.2 and rPAL4.3 could be the result of differences in transformation efficiencies, differences in the fusion between the rice PAL promoter and GUS genes in the constructs, or, most likely, differences in the responsiveness of the two rice PAL promoters to possible other induction factors.

b. Elicitor-Induced GUS Expression

Elicitor-treated protoplasts transformed with rPAL2.2 yielded GUS activities that were an average of 9.8% higher than those of untreated protoplasts transformed with the same construct. No significant increase in GUS activity was seen in elicitor-treated protoplasts transformed with rPAL4.3 relative to untreated protoplasts that had been transformed with this construct. These data suggest that the λrPAL-2 promoter is inducible by the fungal elicitor evaluated herein while the λrPAL-4 promoter is not.

EXAMPLE IX: RICE

PRODUCTION OF TRANSGENIC RICE PLANTS

A. Isolation, Transformation of Protoplasts, and Regeneration of Transgenic Rice Plants

Rice seeds (*Oryza sativa* cv. Sasanishiki) were obtained from National Small Grains Collection, USDA. Callus cultures were initiated according to the protocol of Kyozuka *et al.* (*Mol. Gen. Genet.*, 1987, 206:408-413). The initiated calli were maintained and proliferated on two different media: MS2 medium (Kyozuka *et al.* 1987) and N6 medium (**Rice Table II**). The formulas for these media are given *supra*.

Rice suspension cultures were initiated from the rice calli on two types of suspension media, N6 medium and R2 medium. Rice calli grown on MS2 medium were initiated in R2 medium and rice calli proliferated on N6 medium were initiated in N6 medium. The R2 medium is as follows:

Rice Table VI: Rice Suspension R2 Medium

Constituent	Amount
R2 inorganic salts (Ohira *et al.*, 1973, *Plant Cell Physiol.* 14:113-114).	
MS vitamins (Murashige *et al.* 1962, *Physiol. Plant.* 15:473-497).	
2,4-Dichlorophenoxyacetic Acid	2.0 mg/l
Myo-Inositol	100.0 mg/l
Sucrose	30.0 g/l
pH 5.8	

The initiated cultures were incubated at 27°C in the dark on a gyratory shaker at a speed of about 50 rpm. Rice suspension cultures were subcultured weekly. At each subculture, 2.5 grams fresh weight of cells were transferred to 25 ml fresh suspension media in a 250 ml flask. A 2-fold increase in growth was observed in a seven day growth period.

Thirteen grams fresh weight of 5-day-old suspension cells which were initiated on R2 medium (two-month-old after the initiation) was incubated in 100 ml Protoplast Wash Solution containing 2% cellulase RS, 0.2% pectolyase Y-23 (pH 6.0) at room temperature on a gyratory shaker at a speed of about 50 rpm in the dark for 3 hours and 45 minutes.

The enzyme-protoplast mixture was passed through a 300 mesh tissue sieve to remove debris and centrifuged at about 100 x g for 10 minutes at room temperature. Pelleted protoplasts were washed twice by resuspending in about 35 ml Protoplast Wash Solution and centrifuging at about 100 x g for 10 minutes after each resuspension. Protoplasts were purified as described above for *Oryza sativa* cv. IR-54. (*See* **Rice Example VIII.A.**)

The Percoll-protoplast gradient was centrifuged at about 200 x g for 15 minutes at room temperature. Protoplasts were collected from the top of 25% Percoll Solution and transferred to 30 ml of Protoplast Wash Solution. Protoplasts were washed twice by resuspending in 30 ml Protoplast Wash Solution and centrifuging at about 100 x g for 10 minutes after each resuspension. The protoplast were resuspended in Protoplast Wash Solution to a density of about 3 x 10^7 protoplast per ml for transformation experiments.

PEG-mediated transformation was conducted using the transformation method described above for *Oryza sativa* cv. IR-54.

The protoplasts treated with plasmid DNA were collected by centrifugation at approximately 100 x g for 15 minutes and resuspended in 1 ml of R2 protoplast medium and mixed with an equal volume of the R2 protoplast medium (which was identical to R2 medium except that it contained 0.4M sucrose) containing 2.5% Sea Plaque agarose and transferred to a 35 mm x 15 mm sterile petri dish. Solidified agarose containing the protoplasts were cut into four blocks and transferred to a 100 mm x 150 mm sterile petri dish

containing 15 ml R2 protoplast medium resuspended with about 500 mg 5-day-old Sasanishiki suspension cells which were initiated on N6 medium and sieved through a 30 mesh tissue sieve.

The cultures were incubated at room temperature in the dark on a gyratory shaker at a speed of about 50 rpm.

After 10 days, agarose blocks containing rice protoplasts were transferred to new plates and Sasamishiki suspension cells were completely removed by washing with the R2 protoplast medium. The agarose blocks were cultured in the 100 mm x 150 mm petri dishes containing 20 ml of R2 protoplast medium.

The cultures were incubated at room temperature in the dark on a gyratory shaker at a speed of about 40 rpm.

After 4 days, agarose blocks containing protoplast-derived colonies were transferred to R2 protoplast medium containing 50 μg/ml hygromycin B.

The cultures were incubated at room temperature in the dark on a gyratory shaker at a speed of about 30 rpm.

After 11 days, protoplast-derived rice calli were visible. The agarose blocks containing rice calli were transferred to N6 soft agarose medium (N6 basal medium, 2 mg/l 2,4-dichlorophenoxy acetic acid, 6% sucrose and 0.25% Sigma type I Agarose, pH 5.7) containing 50 μg/ml hygromycin B.

The cultures were incubated at 27°C with 16 hours of light under 4,000 lux light intensity.

After 10 days, rice calli on agarose blocks were about 1 mm diameter and were picked and transferred to N6 medium containing 50 μg/ml hygromycin. This medium was the same as N6 soft agarose medium except that this medium contained 0.5% Sigma Type I Agarose.

After 2 weeks, when rice calli were about 2-3 mm in diameter, they were transferred onto the regeneration medium containing 50 μg/ml hygromycin. The regeneration medium contained N6 inorganic salts, Murashige and Skoog vitamins (Murashige *et al.*, 1962, *Physiol. Plant.* 15:473-497), 6% sucrose, 1% Sigma type I agarose and 2 mg/ml kinetin. When

regenerated shoots were 3 cm or longer, they were rooted in the hormone-free medium containing 50 ug/ml hygromycin B with 1% Sigma type I agarose. The incubation conditions throughout the experiment were the same as above. When regenerated, the transgenic rice plants are used, for example, in an agrichemical screen to identify inducers of these promoters.

RICE EXAMPLE X

USE OF TRANSGENIC RICE PLANTS AS AN AGRICHEMICAL SCREEN

Transgenic rice are propagated by germinating seeds in suitable tissue culture medium containing suitable concentrations of hygromycin to ensure that the PAL-GUS fusion construct is present in all tissues of the transgenic plant. Once the transgenic plantlets have reached a suitable size, *e.g.*, 3-6 inches tall, they are transplanted to soil and maintained under standard greenhouse conditions.

For use in agrichemical screening assays, plants are grown to a size and/or stage which is manageable under greenhouse conditions and susceptible to a pathogen of interest, *e.g., Pyricularia oryzae*. The plants are treated either by foliage spray or soil immersion with a range of concentrations of the unknown chemical or chemicals. In parallel, additional plants are exposed to water as a negative control or to a known elicitor, *e.g.*, probenazole (Iwata, *et al.* Ann. Phytopath. Soc. Japan, 1980, 46: 297-306) as a positive control. Six to twenty four hours following treatment, leaf tissue is removed and assayed for GUS activity using the fluorometric assay of Jefferson, *et al.*, (*Plant Mol. Biol. Rep.*, 1987, 5:387-405). A positive result is indicated by levels of GUS activity significantly higher than those observed with the negative control. A positive result is indicative of, *e.g.*, identifies, chemicals which are capable of inducing rice PAL promoter(s).

EXAMPLE XI: ASSAY
USE OF 3SR
TO IDENTIFY INDUCIBLE PROMOTER FROM RICE GENOMIC DNA

A. 3SR

The 3SR reaction (self-sustained sequence replication) is a method for *in vitro* amplification of specific RNA sequences (Guatelli, *et al.*, 1990. *Proc. Natl. Acad. Sci. USA.* 87:1874-1878). Synthesis of the predominantly RNA product is provided by the action of AMV reverse transcriptase and T7 RNA polymerase in the presence of ribo- and deoxyribonucleotides. The region to be amplified is specified by a pair of DNA primers, one of which contains a T7 RNA polymerase binding site and is complementary (antisense) to the target mRNA. The second primer is identical to the mRNA (sense) in a region approximately 200-300 bp away from the first primer.

The 3SR technique is illustrated in Figure 3. According to the technique, a target RNA sequence is initially transformed into an RNA/DNA duplex by reverse transcriptase in the region specified by the first primer. This duplex is attacked by the RNaseH present on the AMV reverse transcriptase. The RNaseH destroys the RNA template but leaves the cDNA intact. The cDNA is used as a template for the synthesis of a second strand of cDNA using reverse transcriptase and the second primer. The resulting double stranded DNA template serves as a substrate for T7 RNA polymerase to produce multiple copies of antisense RNA which will hybridize to primer 2 to start a second cycle of double-stranded cDNA synthesis. This again results in RNA transcription, and the antisense RNA permits the recycling of the 3SR reaction until some limitation in the reaction stops the cycling. The target RNA may be amplified 10^6 - 10^9 fold.

This example illustrates use of the 3SR technique to identify inducible promoters from bean genomic DNA.

B. Tissue Culture Conditions

Bean (*Phaseolus vulgaris* cv. Canadian Wonder) suspension cultures were obtained from Dr. C. Lamb. The cultures were maintained and proliferated on modified Schenk and Hildebrandt medium (SH medium) (*infra*,

Assay Table VII). The cultures were transferred weekly. At each subculture, six grams fresh weight of bean suspension cells were transferred to 100 ml fresh SH medium. The cultures were incubated at 27°C in the dark on a gyratory shaker at a speed of 125 rpm.

C. Amplification of Bean PAL-1

PAL induction experiments were conducted on bean cell suspension cultures seven days after subculture. Two gram aliquots of the cultures were treated for three hours with the following conditions: (1) 20 μg/ml of fungal elicitor isolated from the walls of *Colletotrichum lindemuthianum* race Alpha according to **Rice Table IV**; (2) *Escherichia coli* strain DH5a (BRL, Bethesda, MD); (3) *Agrobacterium tumefaciens* strain C58 (Dr. Maarten Chrispeels, UCSD, LaJolla, CA); (4) *Clavibacter michiganese* pv *michiganese* (American Type Culture Collection, Rockville, MD); (5) *Xanthomonas campestris* pv *malvacearum* (ATCC, Rockville, MD); (6) *Pseudomonas syringae* pv *tomato* (ATCC, Rockville, MD); (7) *Pseudomonas syringae* pv *tabaci* (ATCC, Rockville, MD); (8) *Erwinia carotovora* subsp. *carotovora* (ATCC, Rockville, MD); (9) SH medium (negative control, t=3); and (10) no treatment, immediately frozen (t=0). All bacteria were added to a concentration of approximately 10^7 - 10^8 cells/ml.

Total nucleic acid was isolated from 0.2 - 0.3 grams of each sample using the following protocol: (1) frozen tissue was ground to a fine powder in a mortar and pestle, (2) pulverized tissue was added to 450 μl NT buffer (0.1M NaCl, 0.01M Tris, pH9.0, 1mM EDTA) and 450 μl phenol:chloroform (1:1) and vortexed until thawed, (3) the slurry was centrifuged for 10 min. in a microfuge, (4) the aqueous layer was removed and precipitated with EtOH according to standard protocols in Maniatis *et al.*, (1982), (5) the nucleic acids were recovered by centrifugation and the concentration determined by spectrophotometry at 260nm, (6) the final concentration was adjusted to 0.6 μg/ml.

The total nucleic acid was subjected to 3SR amplification using primers derived from the sequence of the bean PAL-1 gene (Edwards, *et al.*, 1985. *Proc. Natl. Acad. Sci. USA.* 82:6731-6735.) The sequence of the primers

is given in **SEQ ID NOS. 10** and 12. (The T7 RNA polymerase binding site on Primer 1 is given in **SEQ ID NO. 11.**) Total nucleic acid at a concentration of approximately 0.6 μg was used for the 3SR reaction, and the exact details are given in **Assay Table VIII.**

The 3SR reaction products were analyzed using a dot blot apparatus (Schleicher and Schuell, Keene, NH). Two microliters of the reaction was added to 100 microliters of DM5 (2.6mM Tris, pH 8.0, 0.26mM EDTA, 10 x SSC, 7.4% Formaldehyde), and the samples were heated to 55°C for 20 minutes, then placed on ice prior to loading onto the nitrocellulose in the dot blot apparatus according to manufacturer's instructions. The nucleic acids were fixed to the nitrocellulose by UV crosslinking (Stratagene, LaJolla, CA). The blot was probed with an oligonucleotide derived from the bean PAL-1 gene (Edwards *et al.*, 1985, *supra*). The sequence was identical to the mRNA (sense) strand. The probe is shown in **SEQ ID NO. 13.** The oligonucleotide was end-labeled with gamma $_{32}$P-ATP using T4 kinase (BRL, Bethesda, MD) according to Maniatis *et al.*, 1982, *supra.* The blot was hybridized with 2 x 10^6 cpm/ml for 1 hour in 5 x SSPE, 4 x BP (2% BSA, 2% Polyvinylpyrrolidone-40), 1% SDS. The filter was washed three times at room temperature for 5 minutes each in 1 x SSPE, 1% SDS and one time at 42°C for 1 minute in 1 x SSPE, 1% SDS.

More PAL hybridizable material was detected with the induced samples than with the uninduced samples. Therefore this data suggests that PAL-1 is a gene inducible by plant pathogenic bacteria. In addition, it could be concluded that 3SR™ is a rapid and sensitive technique which can be used for the identification of inducers of plant defense genes.

Assay Table VII: Schenck and Hildebrant SH Medium

Constituent	Amount, mg/l
KNO_3	2500.00
NaH_2PO_4	300.00
$MgSO_4 \cdot 7H_20$	400.00
$CaCl_2 \cdot 2H_20$	225.00
Na_2-EDTA	20.00

$FeSO_4 \cdot 7H_20$	13.90
$MnSO_4 \cdot H_20$	12.50
H_3BO_3	5.00
$ZnSO_4 \cdot 7H_20$	1.00
KI	1.00
$CuSO_4 \cdot 5H_20$	0.20
$Na_2MoO_4 \cdot 2H_20$	0.10
Thiamine · HCl	15.00
Pyridoxine · HCl	1.50
Nicotinic Acid	15.00
2,4-Dichlorophenoxyacetic Acid	0.440
P-Chlorophenoxyacetic acid	2.100
Kinetin	0.105
Sucrose	34,000.00
Myo-Inositol	1,000.00
pH 5.8	

Assay Table VIII

X: 3SR Protocol

5x Reaction Buffer	20.0 µl
200 mM Tris-HCL, pH 8.1	
150 mM $MgCl_2$	
100 mM KCl	
20 mM Spermidine	
50 mM Dithiothreitol	
dNTP's (25 mM) (Promega, Madison, WI)	4.0 µl
rNTP's (25 mM) (Aldrich, Milwaukee, WI)	20.0 µl
DMSO (Sigma, St. Louis, MO)	10.0 µl
Sorbitol (66.7% w/v) (Sigma, St. Louis, MO)	22.5 µl

Primer 1	5.0 μl
Primer 2	5.0 μl
Water	8.5 μl
Target nucleic acid	5.0 μl

Heat sample at 65°C, 1 min

Transfer to 37°C, 1 min

Place on ice

Add 1 μl AMV Reverse Transcriptase (20 u) (Life Sciences, St. Petersburg, FL)

1 μl T7 RNA Polymerase (50 u) (Stratagene, LaJolla, CA)

Reaction is placed at 42°C for 1 hour

Stop amplification by placing on ice

CONCLUSION

Without departing from the spirit and scope of this invention, one of ordinary skill can make various changes and modifications to the invention to adapt it to various usages and conditions. As such, these changes and modifications are properly, equitably, and intended to be, within the full range of equivalence of the following claims.

SUMMARY OF SEQUENCES

Sequence ID NO. 1:	pPAL-1, 5′→ 3′, B ↔ S fragment; 245 bp sequence
Sequence ID NO. 2:	pPAL-2, 5′→ 3′, (E) ↔ P fragment; 271 bp sequence
Sequence ID NO. 3:	pPAL-3, 5′→ 3′, (E) ↔ P frament; 277 bp sequence
Sequence ID NO. 4:	pPAL-4, 5′→ 3′, E ↔ P fragment; 276 bp sequence
Sequence ID NO. 5:	pPAL-6(a), 5′→ 3′ H ↔ S fragment; 300 bp sequence
Sequence ID NO. 6:	pPAL-6(b), 5′→ 3′, H ↔ S fragment; sequenced S → H, *i.e.*, antisense, beginning at *Sst*I and

continuing towards *Hin*dIII site antisense sequence converted to sense sequence; 260 bp sequence

Sequence ID NO. 7: pPAL-8, 5'→ 3' (E) ↔ P fragment; 278 bp sequence

Sequence ID NO. 8: rPAL-2, 5'→ 3'; 2338 bp sequence, sense strand, promoter and partial structural gene coding sequence

Sequence ID NO. 9: rPAL-4, 5'→ 3'; 1997 bp sequence, sense strand, promoter and partial structural gene coding sequence

Sequence ID NO. 10: 3SR Primer 1

Sequence ID NO. 11: T7 RNA Polymerase Binding Site on 3SR Primer 1

Sequence ID NO. 12: 3SR Primer 2

Sequence ID NO. 13: Probe from bean PAL-1

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Fitzmaurice Ph.D., Leona C.
Virts Ph.D., Elizabeth L.
Lin, Fen-Fen
Mirkov Ph.D., T. Erik
Collier, Jana G.
Schoeneck, Paula

(ii) TITLE OF INVENTION: Inducible Plant Defense Gene Regulatory Sequences from Potato and Rice, Uses Thereof, and Assays

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: McCubbrey, Bartels, Meyer, & Ward
(B) STREET: One Post St.
(C) CITY: San Francisco
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94104-5231

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 635,820
(B) FILING DATE: 02-JAN-1991

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 352,658
(B) FILING DATE: 18-MAY-1989

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 343,466
(B) FILING DATE: 26-APR-1989

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 197,122
(B) FILING DATE: 20-MAY-1988

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Meyer Esq., Virginia H.
(B) REGISTRATION NUMBER: 30089
(C) REFERENCE/DOCKET NUMBER: 51633M

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 391-6665
(B) TELEFAX: (415) 391-6663

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 245 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tuberosum
(B) STRAIN: cv. Desiree

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPAL-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GATCCTCTTC AGAAACCAAA GCAAGCATCG TTATGCTCTC CGAACATCTC CACAATGGCT      60

TGGCCCTCAA ATTGAAGTCA TACGCGCAGC AACTAAGATG ATTGAGAGGG AGATTAACTC     120

AGTGAACGAC AATCCATTGA TCGATGTATC AAGAAACAAG GCCTTGCACG GTGGCAACTT     180

TCAAGGCACC CATATGGTGT GTCATGGATA ATACAGATTG GCCTGCATCA TAGGAATGAT     240

GTTTG                                                                245
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 271 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tuberosum
(B) STRAIN: cv. Desiree

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPAL-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
CTTGGGATTA ATCTCAGCCA GGAAAACAGC TGAGGCTGTT GATATCTTGA AGCTAATGTC      60

ATCAACCTAT CTCGTGGCGC TTTGCCAAGC TATAGACTTA CGGCATTTGG AGGAGAACTT     120

GAAGAGTGCT GTCAAGAACA CAGTTAGCCA AGTAGCTAAG AGAACTTTGA CAATGGGTGC     180

TAATGGGGAA CTTCATCCAG CAAGATTCTG TGAGAAGGAA TTGCTTCGAG TCGTGGATAG     240

GGAATACTTG TTTGCCTATG CAGATGACCC C                                    271
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 277 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tuberosum

(B) STRAIN: cv. Desiree

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPAL-3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GAATTCCTTG GGATTAATCT CAGCCAGGAA AACAGCCGAG GCTGTTGATA TCTTGAAGCT    60
AATGTCATCA ACCTATCTCG TGGCGCTTTG CCAAGCTATA GACTTANNGC ATTTGGAGGA   120
GAACTTGAAG AGTGCTGTCA AGAACACAGT TAGCCAAGTA GCTAAGAGAA CTTTGACAAT   180
GGGTGCTAAT GGTGAACTTC ATCCAGCAAG ATTCTGCGAA AAGGAATTGC TTCGAGTCGT   240
GGACAGGGAA TACTTGTTTG CCTATGCAGA TGACCCC                            277
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 276 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tuberosum
(B) STRAIN: cv. Desiree

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPAL-4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GAATTCCTTG GGCTTAATCT CAGCCAGGAA AACAGCTGAG GCTGTTGATA TCTTGAAGCT    60
AATGTCATCA ACCTATCTCG TGGCGCTTTG CCAAGCTATA GACTTACGGC ATTTGGAGGA   120
GAACTTGAAG AGTGCTGTCA AGAACACAGT TAGCCAAGTA GCTAAGAGAA CTTTGACAAT   180
GGGTGCTAAT GGTGAACTTC ATCCAGCAAG ATTTTGCGAA AAGGAATTGC TTCGAGTCGT   240
GGACAGGGAA TACTTGTTTG CCTATGCAGA TGACCC                             276
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 300 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tuberosum
(B) STRAIN: cv. Desiree

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPAL-6a

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
AAGCTTGGAC TATGGTTTCA AGGGAGCTGA AATCGCGATG GCTTCTTACT GCTCGGAACT   60
TCAATTCTTG GCAAATCCAG TGACCAACCA TGTTCAGAGT GCCGAGCAAC ACAACCAAGA  120
TGTGAACTCC TTAGGCTTAA TCTCAGCAAG GAAAACAGCT GAGGCTGTCG ACATCTTAAA  180
GCTAATGTCA TCAACCTATC TCGTGGCACT TTGCCAAGCT ATAGACTTGA GGCATTTGGA  240
GGAGAACTTG AAGAGTGTTG TCAAGAACAC AGTTAGCCAA GTAGCTAAGA GACTTTGACA  300
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 260 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tuberosum
(B) STRAIN: cv. Desiree

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPAL-6b

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
TGGTGAACTT CATCCAGCAA GATTCTGCGA GAAGGAATTG CTTCGAGTCG TAGACAGGGA      60
ATACTTGTTT ACCTATGCTG ATGACCCCTG CAGCTCCACC TATCCTTTGA TGCAGAAGCT     120
GAGACAGGTC CTTGTTGATC ATGCAATGAA GAATGGTGAA AGTGAGAAGA ATATCAACAG     180
CTCAATCTTC CAAAAGATTG GAGCTTTCGA GGACGAATTA AATGCTGTGT TGCCTAAAGA     240
AGTTGAGAGT GCAAGAGCTC                                                 260
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 278 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tuberosum
(B) STRAIN: cv. Desiree

(vii) IMMEDIATE SOURCE:
(B) CLONE: pPAL-8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
GAATTCCTTG GGATTAATCT CAGCCAGGAA AACAGCCGAG GCTGTCGATA TCTTGAAGCT      60
AATGTCATCA ACCTATCTCG TGGCGCTTTG CCAAGCTATA GACTTGAGGC ATTTGGAGGA     120
AAACTTGAAG AGTGCTGTCA AGAACACAGT TAGCCAAGTA GCTAAGAGAA CTTTGACAAT     180
GGGTGCTAAT GGTGAACTTC ATCCAGCAAG ATTCTGCGAA AAGGAATTGC TTCGAGTCGT     240
```

GGACAGGGAA TACTTGTTTG CCTATGCAGA TGATCCCC 278

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2338 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryza sativa

(vii) IMMEDIATE SOURCE:
(B) CLONE: lambda rPAL-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCTCCTTGT CCCGGCCAAG TCGGCCGAGC TCGAATTCCA ATGATAGTTA TGTTTGATTT 60

TTACAATCCC AATGACAATA AAGAGTATGC GGCGGACGAG TCGTAGAGGA GTATAGTGGC 120

AGTGTTTGAC GGGTTTTCTA AAAATTATAA AAAACATGAA ACCCAACGAG ACAATAAACT 180

CTAAAAACTA TAAGGTTCAA ATTTTAAAGG TTCGGGCTTC TAAAAAATAA AAAAATAAAC 240

CCTAATGATA ATCATATTTG ATTTTTAAAA TCTCAATGAC AATAAAGAAG GGCGGCAGCG 300

GGCGGGCCGT AGAGGAGTAC AGTGGCAAAG CAACTGACGG TTTGGCAGGA CTTCTAGAAA 360

GTAAAAAATG AACCCGAATG ATAATTATGT TCGATTTTTA AAATCCCAAT GATAATAAAT 420

AGAAAAGACA ATTGACGGGG CATAGAGGAG TATAATGACA GCGTTTGACG GGACTTATAG 480

AAATTATAAA AACGAAACCC AACGAGACAC TAAACTCTAA AAACTATAAG ATCCTATTTT 540

TAAAGGTTTC AAGGAGAATG AATAGAAATA GTGGTAGATT GAGCAAGCAA ATAAAAAATG 600

ATATGAGAAA AGTAAGACGT AGCAGCTGGT GTGACTTTAA AAACCATATA ATTAGAAATA 660

TGGAGATGAT AAGGTTTGGT CTTTCAAAGT CTTAAGACAA CGAAATAGCT ATTTAATAAA 720

TTTTAAGCAA AATCATACTT AAAAAATATA TAATTTTGTT TGTGTACTAG CCGCGCAGTT 780

GCGCGGGCCA CCAGCTAGTT GAGAGTATAA TTAACTTTTT TTCTTTAAAA TATACACAAT 840

AAACTATATT TTTTAAAAGA TTTTCTGTCG CACAGACATT ATACTAGTTC TGAGAAAAAA 900

CTGTCTATAT TTTCTCAGTC AAGTCAGGTG TGTATTGCGC AGAACGAAAG CTCGGAGAGA 960

ATACTAGCAC TTGGTATGAA CCATTAGGAC TTGCTAAAGA CAGATGAAAG GGTTATGCCA 1020

ACACCAGTTT TTGTCGGGGG ACTGGTGACC GCGCAATTGG ATTGGTACCT CTCTTGTGCC 1080

GTAGTTTCCC CCCTCATGCA CCCCCAAAAC CCCAGAAAAA TTTTGTTGTT TGTACACAGA 1140

CCGCTTGACC ATCAGCCCAT CACCTACGTG CGGAGAACCA ATGACCTATG GAATATGTAA 1200

CTAGAACACA AAACCTAAAC AAACGTGTTG CACGAAGTAG AAAGCGATAG AGAAAGATAA 1260

GCCGGGGGAC CAAGGAAATG ATATCTGGAT ATGAGTTCAC AGCCCTTCCA GATAAACGGA 1320

CGCCCGGACG AAACGAAAGA CGACGAGTCG AGGACCGTCA GCAGCCGCAG AAACGGCGAG 1380

AGACGCCCCC AAGCCAAACG TGGCTTCGTG GCGTGACGAC GCGAGGTGTT TCACGCCCCG 1440

TATCCCCCCG CGCCGCGCTG CCGCGTGCAA CTCTCTCTCT CTTCCCCCGC ATGCACTCCC 1500

GCCACTGCCC GCCGCCCGCA TCGCTCCGCT CCCCCGAGCC CAACCGCCAC AGGGCACGCC 1560

ACGACCACCA CGAAACCTCT ACGTAGCCAC ACGCCCACCC GGCCCGTAGT TGCGGTCCCA 1620

AACTCGTCGC GCCGGCACAC CAATCCCGTG GTCAACCCAA CCGGCCACAC CGAACCCACA 1680

CTCCCCACTC CCACCCATCC TGCGCCTCCT ATTTAAACTC CCCACAACTC CCTCCATTCC 1740

CCTCCAAGAG CAAAGCCACT GCAGCTTCCA TATCCCCGGC TCTTCCGCAC ACACAACTCC 1800

TCCACCTCCA TCGGGAGCAA ACCGCTCGAG CAACCACCAC TCGTTACAGC TAGACATCGA 1860

TCTCCCCTCT CGTTCGCCGT TCCGATGGAG TGCGAGAACG GGCACGTCGC CCCCGCCGCC 1920

AACGGCAGCA GCCTGTGCGT GGCTAAGCCG CGTGCCGACC CGCTCAACTG GGGGAAGGCG 1980

GCGGAGGAGC TGTCCGGGAG CCATCTGGAC GCGGTGAAGC GCATGGTGGA GGAGTACCGC 2040

AGGCCCGTGG TGACGATCGA GGGCGCCAGC CTGACCATCG CGCAGGTCGC GGCGGTGGCC 2100

TCCGCCGGCG CCGCCAGGGT GGAGCTCGAC GAGTCCGCCC GCGGCCGCGT CAAGGCCAGC 2160

AGCGACTGGG TCATGAACAG CATGATGAAC GGCACCCACA GCTACGGCGT CACCACCGGC 2220

TTCGGCGCCA CCTCCCACCG GAGGACCAAG GAGGGCGGCG CGCTCCAGCG AGAGCTTATC 2280

CGGTAAGAAG CCGCAAGAGT TTGCTGTTCG TCTGGTGAGA GCTTGTGTGG ATCAGAGG 2338

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2001 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryza sativa

(vii) IMMEDIATE SOURCE:
(B) CLONE: lambda rPAL-4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
TGTCCGTCGA CCCAGATCTG GGTCGACCTG CAGGTCAACG GATCTTCTAC TGGCGTTCCA      60
CCTGCAAGTA CCTTTTTTCT AAGTAGTGCT AGCTAGCATA CATCCACCAC GTGAGCCGCC     120
GGGGCAGTGG GATGCCGACG TCGCGCACAA CAACATCAAG AGCACGAACA TACTGCTCGC     180
AAAGCCGGCA AGGCATGGCT CGCGGACTAC GGCCTGGCGC GGGTGGTCTC GTCCCTACGG     240
GCCGCCGCGA GCTCGGCGTG GTACCGGCCC CCGACGCACC CCCGGTGCCA CGGGCGTGGG     300
CGTCGAAGAA GGGCGACGTG TACGCGTTCG GCGTGGTGCT CTCGTGCCCG GGCAAGCGAG     360
CTTCCCAACG CGACGCCGCC GTGCCCACCA GCTGCGGCCA CGGTTGCGTC ATCCCCCTTG     420
GCGAAACCAG CGGGCACAGC AACAGCAGCG GGGAAAAGAT GAGCCGGGCA GCTGTGGTAG     480
CAATTGTGGC CGGGGATTTC GCCGGTACAG GTTGAGGCGA TACAGAAGAA GAGAGGAGAG     540
AGAAGGAAGA AGATGGAAAA TGAAGAAGAT GATGGAAAAC GTGATGGTAG TGGTATGATC     600
GTAGTTTTGT AAAATTTCGA TGGCACGACT ACGAATAGAT AAATTTAATT ATAATGGTAT     660
TTTTCTGAAT AGACAAATTT ACAATGGCAT GGACCAATTA ACCCTACCTC TTTCCCATGT     720
GGAGAGTATG CAAGCATGCA ACAACTAGAA AAGATACTCA TGATAGTTAA ACTCCAAATA     780
GTTTTTTCTT GCAAATTACA TATCCGATCA ACAATCCGAT TATACCATTG TATTCGTTGT     840
AATTAAATCT TATAACAAGA TCTTACAAAG ATTATATTTT GATAAAAAAA ATTATATATG     900
TTGTAATTAA TTATATATGT AAGTTACTTT ATCGTATATA TAAATTACTT CTAGATTTAA     960
TTAAATTATA TTTTGGACAT TTGAAAATTT TATTTTATTG GTTAGTTCTA TAATGTGTTC    1020
ATTAAATTTA ATTTCTAGAC AGTCTTATGT TTTTATCCCC ATAATAGATT TGTTTATAAT    1080
```

GTCGTGACAA GTTACTTTAT TTTTATGTTA CTTCTATACT TATATGCAAA TTACATTTAG 1140

GCTTTATTGA ATTTACTTTT ATATGTCTAA GAAGTAATTT AATGAAATCT AAAAATAATT 1200

CAGATATATT ATAAAAGTAA TTTGTATTTT TATAAAAATT ATAGCTATAA ATATTCAATT 1260

GTTACGAACA ATAGTGTTAT CGGATCGTAA ATTGGATGAG TAGTTTAATA GAAATTTTTA 1320

TTTGAATCAT AGGTGGGAGG GATATATTTT TCTACTTGCT TTATGGCCTA GTAGTATCGA 1380

GATAAACATT AAGGCTGTGT TTAGTTCACA CCAAAATTGG AAGTTTGGTT GAAATTGGAA 1440

CGATGTGACG GAAAAGTTAG AAGTTTGTGT GTGTAGGAAA GGTTTTGATG TGATGGAAAA 1500

GTTAGGAAGT TTGAAGAATT ATTTTGTAAC TAAACACGGC GTAAGAGGTC TCTTTGATTT 1560

AGATTTTGCA TAAAACAAGG GACGGTCCCG CTCTTGCTAC TTATTTAAGC ACCCCCCTCA 1620

GTAGTCCTGA CTCCAACAAG CTCCACCGCA AAGATCCTCT GTTAGCTGGA CGACCTGTGG 1680

ACTGCGGTAC GTGGCGCTGC GAGCAATGGA GTGTGAGACC GGTCTGGTCG ACCGTCCCCT 1740

CAACGGCGAC CCCTTGTACT GGGGCAAGGC GGCGGAGGGT CTTGCGGGGA GCCACCTCGA 1800

CGAGGTGAAG AGGATGGTGG TGGAGTACCG CGCGCCCGCT GGTGAAGATC GACGGCGCCA 1860

TGCTCAGCGT CGCCAAGGTG GCAGCCGTCG CTGGCGAGGC CGCCCGGGTG CAGGTGGTGC 1920

TGGACGAATC CGCACGACCC CGCCTGGAGG CTAGTCGCGA GTGGGTCTTC GACAGCACCA 1980

TGAACGGCAC CGACACGTAC A 2001

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: Y

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Phaseolus vulgaris

(vii) IMMEDIATE SOURCE:
(B) CLONE: Primer 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATTTAATAC GACTCACTAT AGAAACAATA GGAAGCCATG GCAATTTCAG CTCC 54

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Synthetic Construct

(vii) IMMEDIATE SOURCE:
(B) CLONE: T7 RNA Polymerase Binding Site on Primer 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AATTTAATAC GACTCACTAT AGAAA 25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Phaseolus vulgaris

(vii) IMMEDIATE SOURCE:

(B) CLONE: Primer 2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGAGAGATT AACTCCGTGA ATGACAACC 29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Phaseolus vulgaris

(vii) IMMEDIATE SOURCE:
(B) CLONE: Probe

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCTACAACAA CGGTCTGCCT TCAA 24

WHAT IS CLAIMED IS:

1. Isolated promoters for plant genes that encode phenylalanine ammonia-lyase (PAL) in potato or rice wherein said promoters are capable of regulating transcription of an associated DNA sequence in suitable hosts and wherein said promoters are inducible, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) and/or wounding.

2. Isolated phenylalanine ammonia-lyase (PAL) gene promoters capable of regulating transcription of an associated DNA sequence in suitable hosts, wherein said promoters are inducible, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) and/or wounding, and wherein said promoters are selected from the group consisting of λpPAL-1, λpPAL-2, λpPAL-3, λpPAL-4, λpPAL-6, λpPAL-7, λpPAL-8, λrPAL-2, λrPAL-4 and λrPAL-10 promoters.

3. Isolated phenylalanine ammonia-lyase (PAL) promoters according to any of Claims 1 or 2 wherein said suitable hosts include plant protoplasts, plant cells, plant callus, plant tissue, developing plantlets, immature whole plants, mature whole plants and seeds.

4. Isolated promoters according to Claim 1 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait.

5. Isolated promoters according to Claim 2 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait.

6. Isolated promoters according to Claim 1 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

7. Isolated promoters according to Claim 2 wherein said promoters are operatively linked to at least one associated DNA sequence that

encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

8. Isolated promoters according to Claim 1 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes a reporter protein selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

9. Isolated promoters according to Claim 2 wherein said promoters are operatively linked to at least one associated DNA sequence wherein said associated DNA sequence(s) encodes reporter protein(s) selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

10. Isolated promoters according to Claim 1 wherein said promoters are inducible by, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) wherein said elicitor(s) is selected from the group consisting of the reduced form of glutathione; the reduced form of homoglutathione; glycan elicitors; hexa(β-D-glucopyranosyl)-D-glucitols; lipid elicitors; arachidonic acid; eicosapentaenoic acid; glycoprotein elicitors; fungal elicitors; abiotic elicitors; mercuric chloride; viruses, fungi; bacteria and insects.

11. Isolated promoters according to Claim 2 wherein said promoters are inducible by, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) wherein said elicitor(s) is selected from the group consisting of the reduced form of glutathione; the reduced form of homoglutathione; glycan elicitors; hexa(β-D-glucopyranosyl)-D-glucitols; lipid

elicitors; arachidonic acid; eicosapentaenoic acid; glycoprotein elicitors; fungal elicitors; abiotic elicitors; mercuric chloride; viruses, fungi; bacteria and insects.

12. Transgenic plant compositions containing any of the isolated promoters of any of Claims 1-2 or 4-10 wherein said transgenic plant compositions include plant protoplasts, plant cells, plant callus, plant tissue, developing plantlets, immature whole plants, mature whole plants, and seeds.

13. Isolated promoters for plant genes that encode phenylalanine ammonia-lyase (PAL) in potato wherein said promoters are capable of regulating transcription of an associated DNA sequence in suitable hosts and wherein said promoters are inducible, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) and/or wounding.

14. Isolated phenylalanine ammonia-lyase (PAL) promoters capable of regulating transcription of an associated DNA sequence in suitable hosts, wherein said promoters are inducible or otherwise directly or indirectly responsive to exogenous elicitor(s) and/or wounding, and wherein said promoters are selected from the group consisting of λpPAL-1, λpPAL-2, λpPAL-3, λpPAL-4, λpPAL-6, λpPAL-7, and λpPAL-8.

15. Isolated phenylalanine ammonia-lyase (PAL) promoters according to any of Claims 13 or 14 wherein said suitable hosts include plant protoplasts, plant cells, plant callus, plant tissue, developing plantlets, immature whole plants, mature whole plants and seeds.

16. Isolated promoters according to Claim 13 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait.

17. Isolated promoters according to Claim 14 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait.

18. Isolated promoters according to Claim 13 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or

arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

19. Isolated promoters according to Claim 14 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

20. Isolated promoters according to Claim 13 wherein said promoters are operatively linked to at least one associated DNA sequence wherein said associated DNA sequence encodes reporter protein(s) selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

21. Isolated promoters according to Claim 14 wherein said promoters are operatively linked to at least one associated DNA sequence wherein said associated DNA sequence encodes reporter protein(s) selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

22. Isolated promoters according to Claim 13 wherein said promoters are inducible by, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) wherein said elicitor(s) is selected from the group consisting of the reduced form of glutathione; the reduced form of homoglutathione; glycan elicitors; hexa(β-D-glucopyranosyl)-D-glucitols; lipid elicitors; arachidonic acid; eicosapentaenoic acid; glycoprotein elicitors; fungal elicitors; abiotic elicitors; mercuric chloride; viruses, fungi; bacteria and insects.

23. Isolated promoters according to Claim 14 wherein said promoters are inducible by, or are otherwise directly or indirectly responsive

to, exogenous elicitor(s) wherein said elicitor(s) is selected from the group consisting of the reduced form of glutathione; the reduced form of homoglutathione; glycan elicitors; hexa(β-D-glucopyranosyl)-D-glucitols; lipid elicitors; arachidonic acid; eicosapentaenoic acid; glycoprotein elicitors; fungal elicitors; abiotic elicitors; mercuric chloride; viruses, fungi; bacteria and insects.

24. Transgenic plant compositions containing any of the isolated promoters of any of Claims 13-14 or 16-22 wherein said transgenic plant compositions include plant protoplasts, plant cells, plant callus, plant tissue, developing plantlets, immature whole plants, mature whole plants, and seeds.

25. Transgenic plant compositions containing any of the isolated promoters of any of Claims 13-14 or 16-22 wherein said transgenic plant compositions are *Solanum tuberosum* plant compositions.

26. Isolated promoters for plant genes that encode phenylalanine ammonia-lyase (PAL) in rice wherein said promoters are capable of regulating transcription of an associated DNA sequence in suitable hosts and wherein said promoters are inducible, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) and/or wounding.

27. Isolated phenylalanine ammonia-lyase (PAL) promoters capable of regulating transcription of an associated DNA sequence in suitable hosts, wherein said promoters are inducible or otherwise directly or indirectly responsive to exogenous elicitor(s) and/or wounding, and wherein said promoters are selected from the group consisting of λrPAL-2, λrPAL-4 and λrPAL-10 promoters.

28. Isolated phenylalanine ammonia-lyase (PAL) promoters according to any of Claims 26 or 27 wherein said suitable hosts include plant protoplasts, plant cells, plant callus, plant tissue, developing plantlets, immature whole plants, mature whole plants and seeds.

29. Isolated promoters according to Claim 26 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait.

30. Isolated promoters according to Claim 27 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait.

31. Isolated promoters according to Claim 26 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

32. Isolated promoters according to Claim 27 wherein said promoters are operatively linked to at least one associated DNA sequence that encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

33. Isolated promoters according to Claim 26 wherein said promoters are operatively linked to at least one associated DNA sequence wherein said associated DNA sequence encodes reporter protein(s) selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

34. Isolated promoters according to Claim 27 wherein said promoters are operatively linked to at least one associated DNA sequence wherein said associated DNA sequence encodes reporter protein(s) selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

35. Isolated promoters according to Claim 26 wherein said promoters are inducible by, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) wherein said elicitor(s) is selected from the group consisting of the reduced form of glutathione; the reduced form of homoglutathione; glycan elicitors; hexa(β-D-glucopyranosyl)-D-glucitols; lipid elicitors; arachidonic acid; eicosapentaenoic acid; glycoprotein elicitors; fungal elicitors; abiotic elicitors; mercuric chloride; viruses, fungi; bacteria and insects.

36. Isolated promoters according to Claim 27 wherein said promoters are inducible by, or are otherwise directly or indirectly responsive to, exogenous elicitor(s) wherein said elicitor(s) is selected from the group consisting of the reduced form of glutathione; the reduced form of homoglutathione; glycan elicitors; hexa(β-D-glucopyranosyl)-D-glucitols; lipid elicitors; arachidonic acid; eicosapentaenoic acid; glycoprotein elicitors; fungal elicitors; abiotic elicitors; mercuric chloride; viruses, fungi; bacteria and insects.

37. Transgenic plant compositions containing any of the isolated promoters of any of Claims 26-27 or 29-35 wherein said transgenic plant compositions include plant protoplasts, plant cells, plant callus, plant tissue, developing plantlets, immature whole plants, mature whole plants, and seeds.

38. Transgenic plant compositions according to any of Claims 26-27 or 29-35 wherein said transgenic plant compositions are *Oryza sativa* or *Oryza indica* plant compositions.

39. A chimeric composition which comprises a first DNA component sequence which is, or has substantial sequence homology to, a non-coding DNA sequence of a naturally occurring PAL gene, wherein said non-coding sequence is capable of regulating transcription of an associated DNA sequence in suitable hosts and is inducible by exogenous elicitor(s) and/or wounding and wherein said non-coding DNA sequence is any of the non-coding DNA sequences of λpPAL-1, λpPAL-2, λpPAL3, λpPAL-4, λpPAL-6/7, λpPAL-8, λrPAL-2, λrPAL-4 or λrPAL-10, and a second DNA component which is, or has substantial sequence homology to, part but not all of a transcribable PAL-encoding DNA sequence with which said first component is associated in the naturally occurring gene.

40. A chimeric composition according to Claim 39 wherein at least one structural gene is contained in frame within said second DNA component such that the expression product is a fused PAL peptide.

41. A chimeric composition according to Claim 40 wherein said structural gene(s) encodes a reporter protein selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

42. Plant tissue, plant or seed transformed with any of the chimeric compositions of any of Claims 39-41.

43. Plant tissue, plant or seed transformed with any of the chimeric compositions of any of Claims 39-41 wherein said compositions contain potato PAL promoter(s) and said plant tissue, plant or seed is *Solanum*.

44. Plant tissue, plant or seed transformed with any of the chimeric compositions of any of Claims 39-41 wherein said compositions contain potato PAL promoter(s) and said plant tissue, plant or seed is *Solanum tuberosum*.

45. Plant tissue, plant or seed transformed with any of the chimeric compositions of any of Claims 39-41 wherein said compositions contain rice PAL promoter(s) and said plant tissue, plant or seed is *Oryza*.

46. Plant tissue, plant or seed transformed with any of the chimeric compositions of any of Claims 39-41 wherein said compositions contain rice PAL promoter(s) and said plant tissue, plant or seed is *Oriza sativa* or *Oriza indica*.

47. A probe comprising at least 10 nucleotides wherein said probe is comprised of deoxyribonucleotides from any of SEQ ID NOS. 1-9, or mRNA corresponding thereto.

48. An antisense probe comprising at least 10 nucleotides wherein said probe is comprised of deoxyribonucleotides complementary to any of SEQ ID NOS. 1-9, or mRNA corresponding thereto.

49. An antisense probe comprising at least 10 nucleotides wherein said probe is comprised of deoxyribonucleotides complementary to any of SEQ ID NOS. 1-9, or mRNA corresponding thereto.

50. A method for initiating transcription of a chimeric composition comprised of at least one PAL promoter from potato or rice wherein said promoter is operatively linked to at least one structural gene, wherein said promoter is inducible by, or is otherwise directly or indirectly responsive to, exogenous elicitor(s), said method comprising: introducing said chimeric composition into a suitable host and subjecting said host to exogenous elicitor(s) capable of directly or indirectly inducing a response by said promoter.

51. A method for initiating transcription of a chimeric composition contained within a transgenic plant wherein said composition is comprised of at least one PAL promoter from potato or rice wherein said promoter is operatively linked to at least one structural gene, and wherein said promoter is inducible by, or is otherwise directly or indirectly responsive to, exogenous elicitor(s), said method comprising: subjecting said transgenic plant to exogenous elicitor(s) capable of directly or indirectly inducing a response by said promoter.

52. A method for identifying elicitor-inducible promoters, said method comprising: a). subjecting a plant to elicitor; b). isolating RNA from said plant from step a) after said plant has been subjected to said elicitor; c). preparing a cDNA library from said RNA from step b); d). probing said library with a probe comprised of nucleotide sequences from the transcribed coding and/or non-coding region of gene(s) of interest; e). using said probe described in step d) or the cDNA which hybridized with said probe from step d) to probe a genomic library from a plant of interest; f). identifying the segment of genomic DNA that hybridized with said probe from step e); g). making and labeling an antisense RNA transcript from the genomic DNA segment of step f); h). using said labeled antisense RNA transcript from step g) as a probe to hybridize to mRNA from elicitor-treated and non-elicitor treated plants; i). subjecting the hybridized mix to RNase; j). identifying those antisense RNA

transcripts that were protected from degradation by elicitor-treated RNA but were not protected from degradation by RNA from non-elicitor treated plants; k). concluding that the promoter from the genomic clone that yielded the antisense RNA transcript that was protected by only RNA from elicitor-treated plants is inducible with elicitor.

53. A method for identifying elicitor-inducible promoters, said method comprising: a). probing a plant genomic library with a probe comprised of nucleotide sequences from the transcribed coding and/or non-coding region(s) of a gene of interest; b). identifying the segment of genomic DNA that hybridized with said probe from step a); c). making and labeling an antisense RNA transcript from the genomic DNA segment of step b); d). using said labeled antisense RNA transcript from step c) as a probe to hybridize to mRNA from elicitor-treated and non-elicitor treated plants; e). subjecting the hybridized mix to RNase; f). identifying those antisense RNA transcripts that were protected from degradation by elicitor-treated RNA but were not protected from degradation by RNA from non-elicitor treated plants; g). concluding that the promoter from the genomic clone that yielded the antisense RNA transcript that was protected by only RNA from elicitor-treated plants is inducible with elicitor.

54. Isolated DNA sequences comprised of any of the DNA sequences of any of SEQ ID NOS. 1-9.

55. Isolated DNA sequences according to Claim 53 wherein said sequences are operatively linked to at least one structural gene encoding sequence and at least one terminator sequence.

56. Isolated DNA sequences according to Claim 54 wherein said structural gene encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

57. Isolated DNA sequences according to Claim 54 wherein said structural gene(s) encodes at least one reporter protein selected from the

group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (β-GAL), and luciferase (LUX).

58. A method for identifying exogenous elicitors which are capable of inducing, either directly or indirectly, a PAL promoter, said method comprising transforming a suitable host with a chimeric DNA sequence of any of Claims 39-41 or a vector containing any of said chimeric DNA sequences, applying putative exogenous elicitor to said transformed host, and identifying as an exogenous elicitor those exogenous elicitor(s) which cause expression of said second DNA component sequence.

59. A whole plant assay system for identifying exogenous elicitors which are capable of inducing, either directly or indirectly, a PAL promoter from potato or rice, said method comprising creating a transgenic plant that contains at least one chimeric DNA sequence comprised of a potato or rice PAL promoter operatively linked to a reporter structural gene, applying putative exogenous elicitor to said transgenic plant, and identifying as an exogenous elicitor those exogenous elicitor(s) which cause expression of said reporter structural gene.

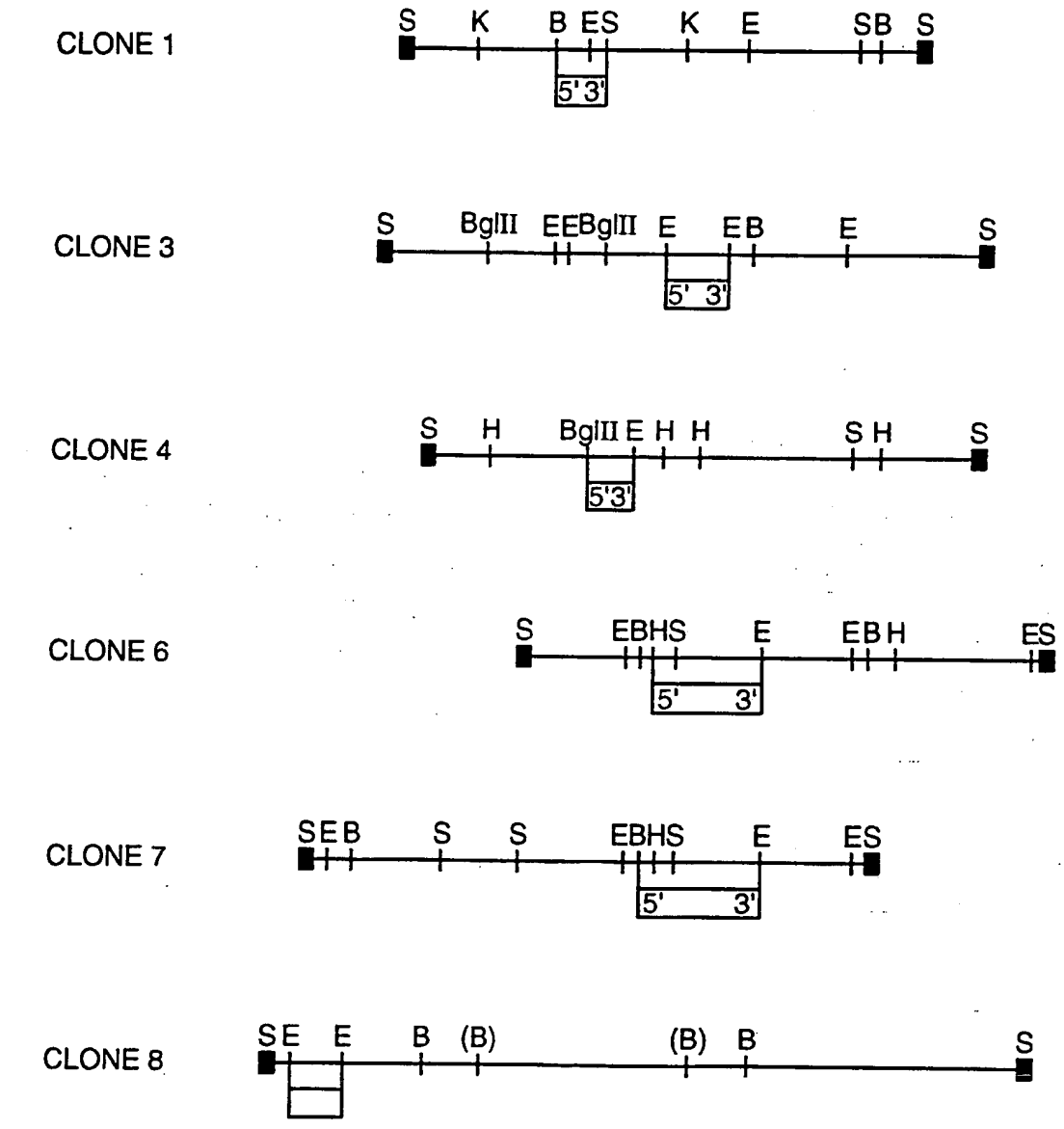
60. A method for identifying organisms which can induce transcription of defense genes, said method comprising exposing a transgenic plant that contains a plant defense gene promoter operatively linked to a reporter gene to an organism of interest, monitoring expression of said reporter gene, and concluding that an organism of interest which can induce transcription of said reporter gene can induce expresssion of plant defense genes, or chimeric constructs that contain regulatory regions or promoters for such plant defense genes.

61. A method for according to Claim 60 wherein said orgainsim is

selected from living organisms, non-living organisms, or living but non-viral or otherwise disabled organisms.

62. A method according to Claim 60 wherein said organism is a virus, a bacteria, a fungus, or an insect.

63. A method for identifying agrichemicals which can induce expression of a plant gene, said method comprising (a) isolating RNA from plant material not exposed to a putative inducer; (b) identifying from within the RNA of step (a) an RNA encoded by a plant gene of interest; (c) amplifying said identified RNA from step (b) using 3SR techniques; (d) exposing to a putative chemical inducer plant material that is identical to the plant material from which the RNA of step (a) was isolated; (e) isolating RNA from said exposed plant material of step (d); (f) identifying from within the RNA of step (e) RNA encoded by same plant gene of interest used to identify the RNA of step (b); (g) amplifying said identified RNA from step (e) using 3SR techniques; (h) comparing the amplification products from steps (c) and (g); (i) identifying as an agrichemical that can induce expression of the gene of interest those agrichemicals which induce a stronger amplification in step (g) than was obtained in step (c).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CLONE 3
S BglII EEBglII E EB E S
5' 3'
CLONE 4
S H BglII E H H S H S
5'3'
CLONE 6
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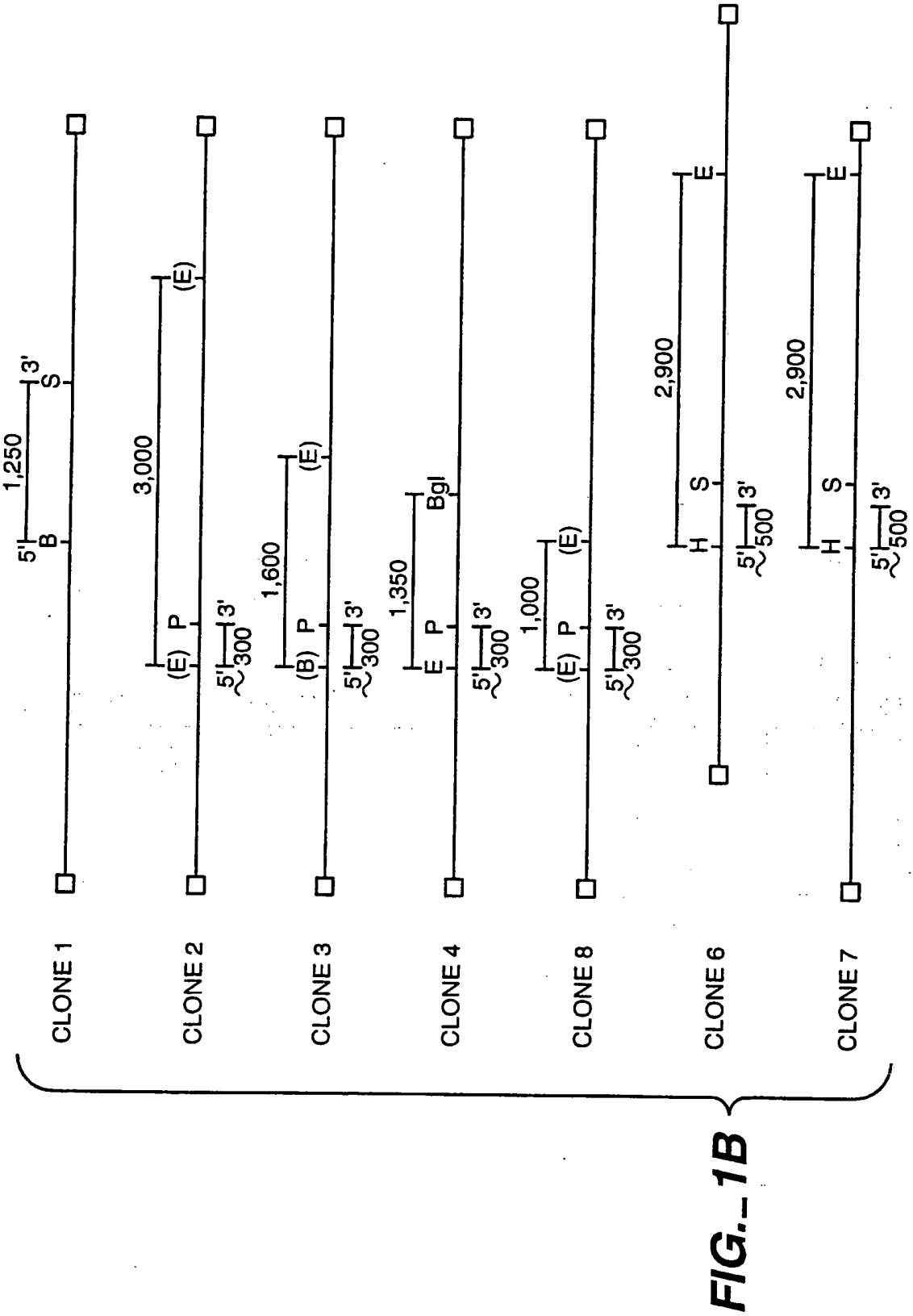
5' 3'
CLONE 7
SEB S S EBHS E ES
5' 3'
CLONE 8
SE E B (B) (B) B S

FIG._1A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S
3'
CLONE 2
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(E)
(E)
P
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300
3'
CLONE 3
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(B)
(E)
P
5'
300
3'
CLONE 4
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E
Bgl
P
5'
300
3'
CLONE 8
1,000
(E)
(E)
P
5'
300
3'
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E
S
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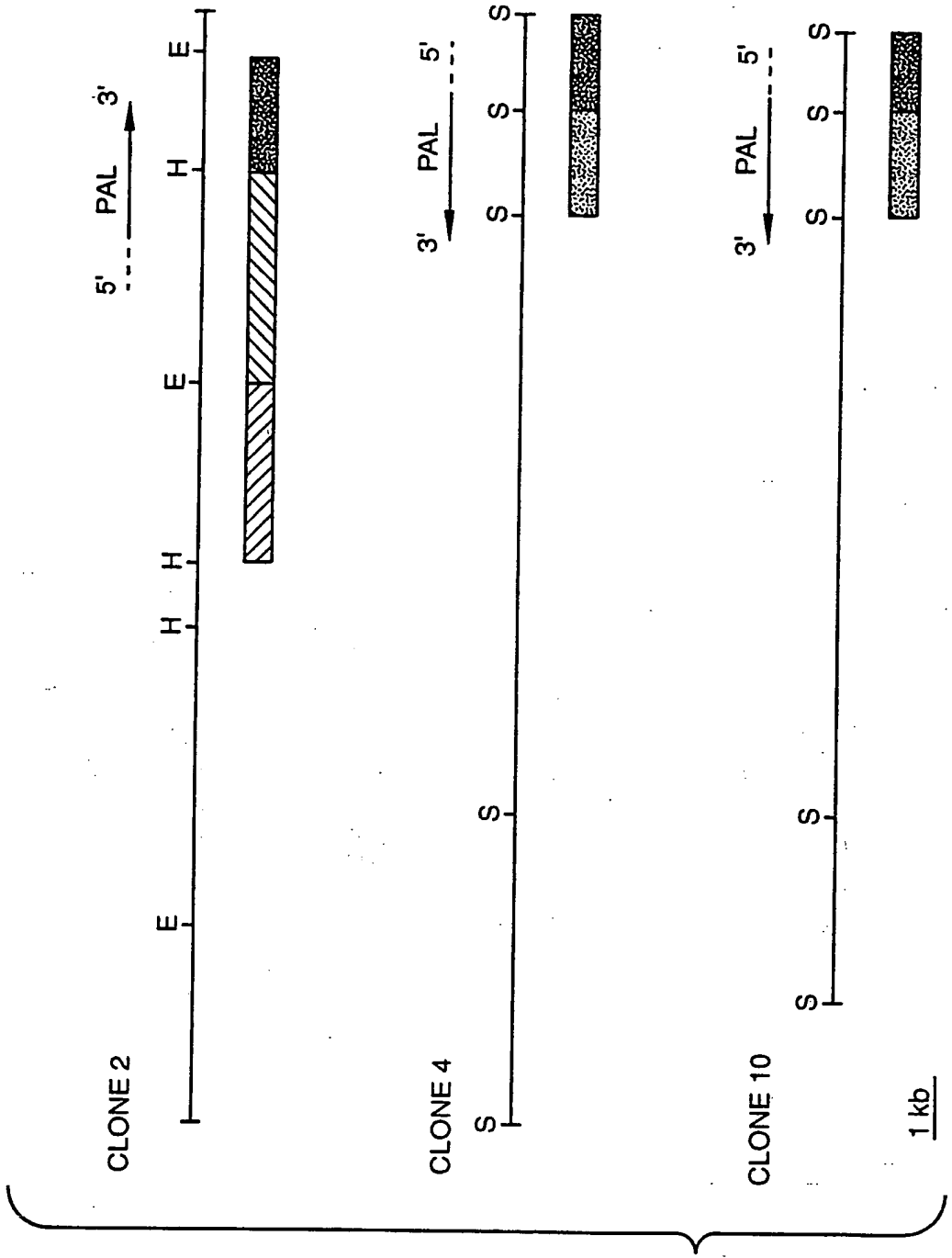
500
3'

FIG._1B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H
H
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CLONE 2
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PAL
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S
S
S
CLONE 10
1 kb

FIG._2

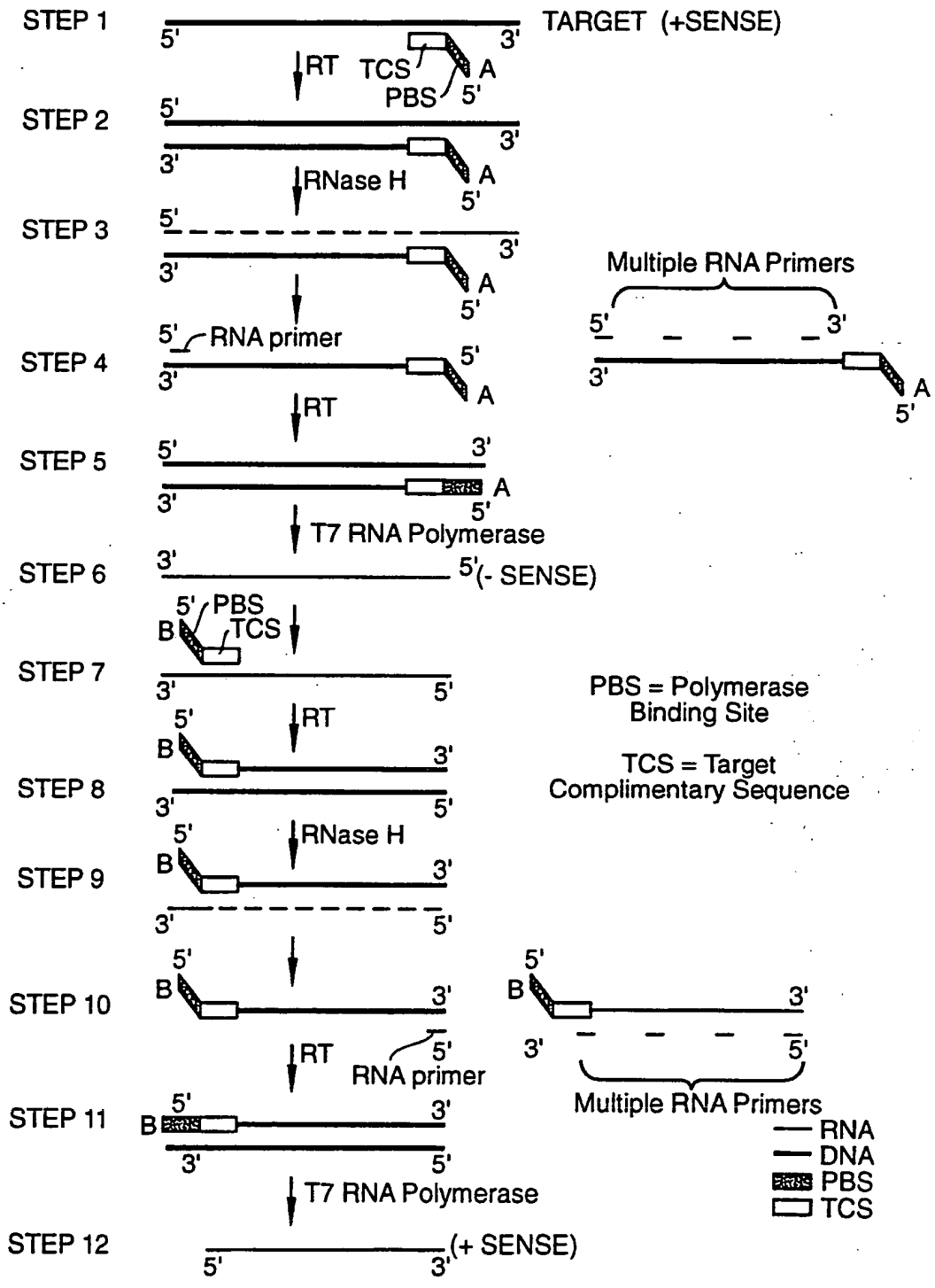

FIG._3

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/08560

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/60; A01H5/00; C12Q1/68
C12Q1/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H ; C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 24, 25 August 1989, BALTIMORE, MD US pages 14486 - 14492 LIANG, X., ET AL. 'Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues'	52-53
Y	see page 14487 ---	49
X	EMBO JOURNAL vol. 8, no. 6, 1989, EYNSHAM, OXFORD GB pages 1641 - 1648 LOIS, R., ET AL. 'A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements'	47,48
Y	see page 1647, right column ---	49
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 FEBRUARY 1993	18. 3. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PLANT MOLECULAR BIOLOGY. vol. 12, 1989, DORDRECHT, THE NETHERLANDS. pages 367 - 383 CRAMER, C.L., ET AL. 'Phenylalanine ammonia-lyase gene organization and structure' cited in the application see figure 2	54
X	EUR. J. BIOCHEM. vol. 185, no. 1, 1989, pages 19 - 25 MINAMI, E., ET AL. 'Structure and some characterization of the gene for phenylalanine ammonia-lyase from rice plants' cited in the application see the whole document	54
X	THE PLANT CELL. vol. 2, December 1990, ROCKVILLE, MD, USA. pages 1131 - 1143 CASTRESENA, C., ET AL. 'Tissue-specific and pathogen-induced regulation of a Nicotiana plumbaginifolia Beta-1,3-glucanase gene' see page 1137, right column	60-62
Y	WO,A,8 912 230 (SALK INSTITUTE) 14 December 1989 see claims 1,6-14	1-34,50, 51,55-59
Y	AGRIC. BIOL. CHEM. vol. 52, no. 4, 1988, pages 943 - 950 HAGA, M., ET AL. 'Dependence on ethylene of the induction of phenylalanine ammonia-lyase activity in rice leaf infected with blast fungus' see the whole document	1-12, 26-34, 50,51, 55-59

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	BIOLOGICAL ABSTRACTS vol. 85 , 1988, Philadelphia, PA, US; abstract no. 20305, FRITZEMEIER, K.-H., ET AL. 'Transient induction of phenylalanine ammonia-lyase and 4-coumarate: coenzyme A ligase messenger RNA in potato leaves infected with virulent or avirulent races of Phytophtora infestans' see abstract & PLANT PHYSIOLOGY. vol. 85, no. 1987, ROCKVILLE, MD, USA. pages 34 - 41	1-25,50, 51,55-59
Y	BIOTECHNOLOGY vol. 8, no. 9, September 1990, NEW YORK US pages 845 - 848 DOERNER, P.W., ET AL. 'Plant defense gene promoter-reporter gene fusions in transgenic plants: tools for identification of novel inducers' see the whole document	59
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 86, December 1989, WASHINGTON US pages 9284 - 9288 LIANG, X., ET AL. 'Developmental and environmental regulation of a phenylalanine ammonia-lyase-Beta-glucuronidase gene fusion in transgenic tobacco plants' see page 9285, left column, line 1 - line 7	55
A	THE PLANT CELL. vol. 2, no. 9, 1990, ROCKVILLE, MD, USA. pages 837 - 848 OHL, S., ET AL. 'Functional properties of a phenylalanine ammonia-lyase promoter from Arabidopsis' see the whole document	1-34, 47-62

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US92/08560

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 35-46
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Pages missing from claims, page 65. Futhermore page 12 missing from description therefore claims 1-34, 47-63 were searched in accordance with the information provided in the incomplete description.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9208560
SA 65697

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 01/02/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8912230	14-12-89	EP-A- 0414809	06-03-91
		EP-A- 0416032	13-03-91
		JP-T- 3504921	31-10-91
		WO-A- 8912059	14-12-89